PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
PAP135-PCT		
International application No.	International filing date (*day/month/year*)	(Earliest) Priority Date (*day/month/year*)
PCT/SG 99/00079	16 July 1999 (16.07.99)	18 February 1999 (18.02.99)
Applicant		
NATIONAL UNIVERSITY OF SINGAPORE et al.		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 3 sheets.

☐ It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**
   a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.
      ☐ the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).
   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing:
      ☒ contained in the international application in written form.
      ☐ filed together with the international application in computer readable form.
      ☐ furnished subsequently to this Authority in written form.
      ☐ furnished subsequently to this Authority in computer readable form.
      ☐ the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
      ☐ the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.
2. ☐ **Certain claims were found unsearchable** (See Box I).
3. ☐ **Unity of invention is lacking** (See Box II).
4. With regard to the **title**,
   ☒ the text is approved as submitted by the applicant.
   ☐ the text has been established by this Authority to read as follows:
5. With regard to the **abstract**,
   ☒ the text is approved as submitted by the applicant.
   ☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.
6. The figure of the **drawings** to be published with the abstract is Figure No.: 7
   ☒ as suggested by the applicant. ☐ None of the figures.
   ☐ because the applicant failed to suggest a figure.
   ☐ because this figure better characterizes the invention.

PATENT COOPERATION TREATY

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
PAP135-PCT		
International application No.	International filing date (*day/month/year*)	Priority Date (*day/month/year*)
PCT/SG 99/00079	16 July 1999 (16.07.1999)	18 February 1999 (18.02.1999)
International Patent Classification (IPC) or national classification and IPC		
IPC[7]: C12N 15/12, 5/16, C12Q 1/66, 1/48		
Applicant		
NATIONAL UNIVERSITY OF SINGAPORE et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examination Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 3 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of _____ sheets.

3. This report contains indications relating to the following items:

   - I. ☒ Basis of the opinion
   - II. ☐ Priority
   - III. ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV. ☐ Lack of unity of invention
   - V. ☒ Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - VI. ☐ Certain documents cited
   - VII. ☐ Certain defects in the international application
   - VIII. ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
18 January 2000 (18.01.2000)	30 January 2001 (30.01.2001)
Name and mailing address of the IPEA/AT Austrian Patent Office Kohlmarkt 8-10 A-1014 Vienna Facsimile No. 1/53424/200	Authorized officer MOSSER Telephone No. 1/53424/437

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/SG 99/00079

I. Basis of the report

1. With regard to the **elements** of the international application:*

☒ the international application as originally filed

☐ the description:
pages _____ , as originally filed
pages _____ , filed with the demand
pages _____, filed with the letter of _____.

☐ the claims:
pages _____ , as originally filed
pages _____ , as amended (together with any statement) under Article 19
pages _____ , filed with the demand
pages _____, filed with the letter of _____.

☐ the drawings:
pages _____ , as originally filed
pages _____ , filed with the demand
pages _____ , filed with the letter of _____.

☐ the sequence listing part of the description:
pages _____ , as originally filed
pages _____ , filed with the demand
pages _____ , filed with the letter of _____.

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:

☐ the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

☐ the language of publication of the international application (under Rule 48.3(b)).

☐ the language of the translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/ or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

☒ contained in the international application in printed form.

☐ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form.

☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. ☐ The amendments have resulted in the cancellation of:

☐ the description, pages _____.

☐ the claims, Nos. _____.

☐ the drawings, sheets/fig _____.

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

** Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as „originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

*** Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.*

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/SG 99/00079

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	1-18	YES
	Claims		NO
Inventive step (IS)	Claims	1-18	YES
	Claims		NO
Industrial applicability (IA)	Claims	1-18	YES
	Claims		NO

Citations and explanations (Rule 70.7)

The documents cited in the search report are merely interesting contributions to the state of the art and do not interfere with novelty and inventive step of the present claims 1-18. The industrial applicability is obvious for the present claims 1-18.

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing: 24 August 2000 (24.08.00)	
International application No.: PCT/SG99/00079	Applicant's or agent's file reference: PAP135-PCT
International filing date: 16 July 1999 (16.07.99)	Priority date: 18 February 1999 (18.02.99)
Applicant: GONG, Zhiyuan et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International preliminary Examining Authority on:

18 January 2000 (18.01.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO
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1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer:

J. Zahra

Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

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NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

APPLIED RESEARCH CORPORATION
Kent Ridge
P.O. Box 1016
Singapore 911101
SINGAPOUR

Date of mailing (day/month/year)
14 November 2000 (14.11.00)

Applicant's or agent's file reference
PAP135-PCT

IMPORTANT NOTIFICATION

International application No.
PCT/SG99/00079

International filing date (day/month/year)
16 July 1999 (16.07.99)

1. The following indications appeared on record concerning:

[X] the applicant [ ] the inventor [ ] the agent [ ] the common representative

Name and Address	State of Nationality	State of Residence
	Telephone No.	
	Facsimile No.	
	Teleprinter No.	

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[ ] the person [ ] the name [ ] the address [ ] the nationality [ ] the residence

Name and Address	State of Nationality	State of Residence
XU, Yianfei HE, Jiangyan YAN, Tie	Telephone No.	
	Facsimile No.	
	Teleprinter No.	

3. Further observations, if necessary:
Please note that the status of the persons in box 2 has changed. They became applicant and inventor for US only instead of applicant only for all designated States except US.

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[X] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
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1211 Geneva 20, Switzerland

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Authorized officer
Christine Carrié

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PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

SURESAN, Sachithananthan
C/O Tan Rajah & Cheah
9 Battery Road, #15-00
Straits Trading Building
Singapore 049910
SINGAPOUR

Date of mailing (day/month/year)
15 February 2001 (15.02.01)

Applicant's or agent's file reference
PAP135-PCT

IMPORTANT NOTIFICATION

International application No.
PCT/SG99/00079

International filing date (day/month/year)
16 July 1999 (16.07.99)

1. The following indications appeared on record concerning:

[ ] the applicant [ ] the inventor [X] the agent [ ] the common representative

Name and Address

APPLIED RESEARCH CORPORATION
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P.O. Box 1016
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Singapore

State of Nationality:

State of Residence:

Telephone No.: 65-7755822

Facsimile No.: 65-7730924

Teleprinter No.:

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[X] the person [X] the name [X] the address [ ] the nationality [ ] the residence

Name and Address

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Singapore 049910
Singapore

State of Nationality:

State of Residence:

Telephone No.:

Facsimile No.:

Teleprinter No.:

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[X] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
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Authorized officer

Maria Victoria CORTIELLO

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Form PCT/IB/306 (March 1994)

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International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification [7] : C12N 15/12, 5/16, C12Q 1/66, 1/48	A1	(11) International Publication Number: **WO 00/49150**
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9900811-2	18 February 1999 (18.02.99)	SG
Not furnished	14 July 1999 (14.07.99)	SG

(71) Applicants *(for all designated States except US)*: NATIONAL UNIVERSITY OF SINGAPORE [SG/SG]; 10 Kent Ridge Crescent, Singapore 119260 (SG). XU, Yianfei [CN/SG]; Intro, National University of Singapore, 10 Kent Ridge Crescent, Singapore 119260 (SG). HE, Jiangyan [CN/SG]; Intro, National University of Singapore, 10 Kent Ridge Crescent, Singapore 119260 (SG). YAN, Tie [CN/SG]; Intro, National University of Singapore, 10 Kent Ridge Crescent, Singapore 119260 (SG).

(72) Inventors; and

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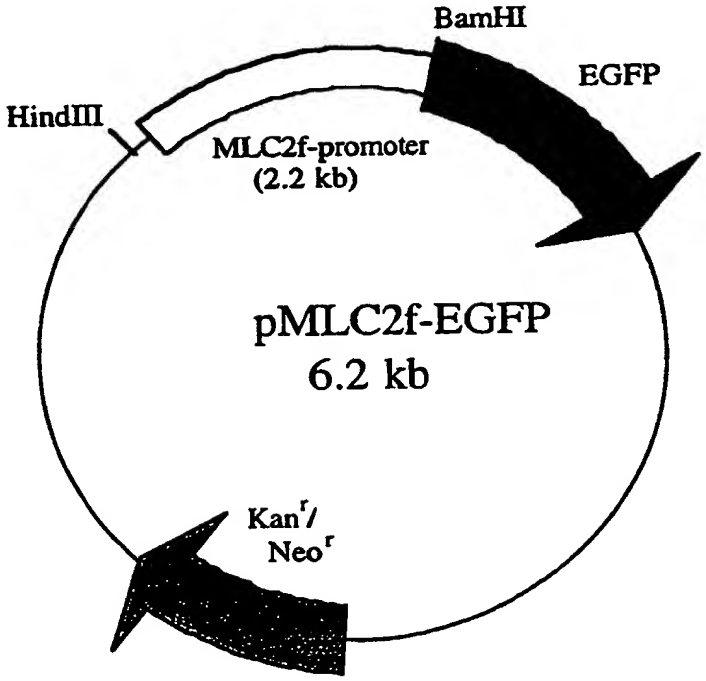
(81) Designated States: CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: CHIMERIC GENE CONSTRUCTS FOR GENERATION OF FLUORESCENT TRANSGENIC ORNAMENTAL FISH

(57) Abstract

Four zebrafish gene promoters, which are skin specific, muscle specific, skeletal muscle specific and ubiquitously expressed respectively, were isolated and ligated to the 5' end of the EGFP gene. When the resulting chimeric gene constructs were introduced into zebrafish, the transgenic zebrafish emit green fluorescence under a blue light or ultraviolet light according to the specificity of the promoters used. Thus, new varieties of ornamental fish of different fluorescence patterns, e.g., skin fluorescence, muscle fluorescence, skeletal muscle–specific and/or ubiquitous fluorescence, are developed.



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CHIMERIC GENE CONSTRUCTS FOR GENERATION OF FLUORESCENT TRANSGENIC ORNAMENTAL FISH

FIELD OF THE INVENTION

This invention relates to fish gene promoters and chimeric gene constructs with these promoters for generation of transgenic fish, particularly fluorescent transgenic ornamental fish.

BACKGROUND OF THE INVENTION

Transgenic technology involves the transfer of a foreign gene into a host organism enabling the host to acquire a new and inheritable trait. The technique was first developed in mice by Gordon et al. (1980). They injected foreign DNA into fertilized eggs and found that some of the mice developed from the injected eggs retained the foreign DNA. Applying the same technique, Palmiter et al. (1982) have introduced a chimeric gene containing a rat growth hormone gene under a mouse heavy metal-inducible gene promoter and generated the first batch of genetically engineered supermice, which are almost twice as large as non-transgenic siblings. This work has opened a promising avenue in using the transgenic approach to provide to animals new and beneficial traits for livestock husbandry and aquaculture.

In addition to the stimulation of somatic growth for increasing the gross production of animal husbandry and aquaculture, transgenic technology also has many other potential applications. First of all, transgenic animals can be used as bioreactors to produce commercially useful compounds by expression of a useful foreign gene in milk or in blood. Many pharmaceutically useful protein factors have been expressed in this way. For example, human αl-antitrypsin, which is commonly used to treat emphysema, has been expressed at a concentration as high as 35 mg/ml (10% of milk proteins) in the milk of transgenic sheep (Wright et al., 1991). Similarly, the transgenic technique can also be used to improve the nutritional value of milk by selectively increasing the levels of certain valuable proteins such as caseins and by supplementing certain new and useful proteins such as lysozyme for antimicrobial activity (Maga and Murray, 1995). Second, transgenic mice have been widely used in medical research, particularly in the generation of transgenic animal models for human disease studies (Lathe and Mullins, 1993). More recently, it has been proposed to use transgenic pigs as organ donors for xenotransplantation by expressing human regulators of complement activation to prevent hyperacute rejection during organ transplantation (Cozzi and White, 1995). The

development of disease resistant animals has also been tested in transgenic mice (e.g. Chen et al., 1988).

Fish are also an intensive research subject of transgenic studies. There are many ways of introducing a foreign gene into fish, including: microinjection (e.g. Zhu et al., 1985; Du et al., 1992), electroporation (Powers et al., 1992), sperm-mediated gene transfer (Khoo et al., 1992; Sin et al., 1993), gene bombardment or gene gun (Zelemin et al., 1991), liposome-mediated gene transfer (Szelei et al., 1994), and the direct injection of DNA into muscle tissue (Xu et al., 1999). The first transgenic fish report was published by Zhu et al. (1985) using a chimeric gene construct consisting of a mouse metallothionein gene promoter and a human growth hormone gene. Most of the early transgenic fish studies have concentrated on growth hormone gene transfer with an aim of generating fast growing "superfish". A majority of early attempts used heterologous growth hormone genes and promoters and failed to produce gigantic superfish (e.g. Chourrout et al., 1986; Penman et al., 1990; Brem et al., 1988; Gross et al., 1992). But enhanced growth of transgenic fish has been demonstrated in several fish species including Atlantic salmon, several species of Pacific salmons, and loach (e.g. Du et al., 1992; Delvin et al., 1994, 1995; Tsai et al., 1995).

The zebrafish, *Danio rerio,* is a new model organism for vertebrate developmental biology. As an experimental model, the zebrafish offers several major advantages such as easy availability of eggs and embryos, tissue clarity throughout embryogenesis, external development, short generation time and easy maintenance of both the adult and the young. Transgenic zebrafish have been used as an experimental tool in zebrafish developmental biology. However, despite the fact that the first transgenic zebrafish was reported a decade ago (Stuart et al., 1988), most transgenic zebrafish work conducted so far used heterologous gene promoters or viral gene promoters: e.g. viral promoters from SV40 (simian virus 40) and RSV (Rous sarcoma virus) (Stuart et al., 1988, 1990; Bayer and Campos-Ortega, 1992), a carp actin promoter (Liu et al., 1990), and mouse homeobox gene promoters (Westerfield et al., 1992). As a result, the expression pattern of a transgene in many cases is variable and unpredictable.

GFP (green fluorescent protein) was isolated from a jelly fish, *Aqueous victoria.* The wild type GFP emits green fluorescence at a wavelength of 508 nm upon stimulation with ultraviolet light (395 nm). The primary structure of GFP has been elucidated by cloning of its cDNA and genomic DNA (Prasher et al., 1992). A modified GFP, also called EGFP (Enhanced Green Fluorescent Protein) has been generated artificially and it contains mutations that allow the protein to emit a stronger green light and its coding sequence has also been optimized for higher expression in mammalian cells based on preferable human

codons. As a result, EGFP fluorescence is about 40 times stronger than the wild type GFP in mammalian cells (Yang et al., 1996). GFP (including EGFP) has become a popular tool in cell biology and transgenic research. By fusing GFP with a tested protein, the GFP fusion protein can be used as an indicator of the subcellular location of the tested protein (Wang and Hazelrigg, 1994) . By transformation of cells with a functional GFP gene, the GFP can be used as a marker to identify expressing cells (Chalfie et al., 1994). Thus, the GFP gene has become an increasingly popular reporter gene for transgenic research as GFP can be easily detected by a non-invasive approach.

The GFP gene (including EGFP gene) has also been introduced into zebrafish in several previous reports by using various gene promoters, including Xenopus *elongation factor 1α* enhancer-promoter (Amsterdam et al., 1995, 1996), rat *myosin light-chain* enhancer (Moss et al., 1996), zebrafish *GATA-1* and *GATA-3* promoters (Meng et al., 1997; Long et al., 1997), zebrafish *α-* and *β-actin* promoters (Higashijima et al., 1997), and tilapia *insulin-like growth factor I* promoter (Chen et al., 1998). All of these transgenic experiments aim at either developing a GFP transgenic system for gene expression analysis or at testing regulatory DNA elements in gene promoters.

SUMMARY OF THE INVENTION

It is a primary objective of the invention to clone fish gene promoters that are constitutive (ubiquitous), or that have tissue specificity such as skin specificity or muscle specificity or that are inducible by a chemical substance, and to use these promoters to develop effective gene constructs for production of transgenic fish.

It is another objective of the invention to develop fluorescent transgenic ornamental fish using these gene constructs. By applying different gene promoters, tissue-specific, inducible under different environmental conditions, or ubiquitous, to drive the GFP gene, GFP could be expressed in different tissues or ubiquitously. Thus, these transgenic fish may be skin fluorescent, muscle fluorescent, ubiquitously fluorescent, or inducibly fluorescent. These transgenic fish may be used for ornamental purposes, for monitoring environmental pollution, and for basic studies such as recapitulation of gene expression programs or monitoring cell lineage and cell migration. These transgenic fish may be used for cell transplantation and nuclear transplantation or fish cloning.

Other objectives, features and advantages of the present invention will become apparent from the detailed description which follows, or may be learned by practice of the invention.

Four zebrafish gene promoters of different characteristics were isolated and four chimeric gene constructs containing a zebrafish gene promoter and EGFP DNA were made: pCK-EGFP, pMCK-EGFP, pMLC2f-EGFP and pARP-EGFP. The first chimeric gene construct, pCK-EGFP, contains a 2.2 kbp polynucleotide comprising a zebrafish cytokeratin (CK) gene promoter which is specifically or predominantly expressed in skin epithelia. The second one, pMCK-EGFP, contains a 1.5 kbp polynucleotide comprising a muscle-specific promoter from a zebrafish muscle creatine kinase (MCK) gene and the gene is only expressed in the muscle tissue. The third construct, pMLC2f-EGFP contains a 2.2 kpb polynucleotide comprising a strong skeletal muscle-specific promoter from the fast skeletal muscle isoform of the myosin light chain 2 (MLC2f) gene and is expressed specifically or predominantly in skeletal muscle. The fourth chimeric gene construct, pARP-EGFP, contains a strong and ubiquitously expressed promoter from a zebrafish acidic ribosomal protein (ARP) gene. These four chimeric gene constructs have been introduced into zebrafish at the one cell stage or two cell stage by microinjection. In all cases, the GFP expression patterns were consistent with the specificities of the promoters. GFP was predominantly expressed in skin epithelia with pCK-EGFP, specifically expressed in muscles with pMCK-EGFP, specifically expressed in skeletal muscles with pMLC2f-EGFP and ubiquitously expressed in all tissues with pARP-EGFP.

These chimeric gene constructs are useful to generate green fluorescent transgenic fish. The GFP transgenic fish emit green fluorescence light under a blue or ultraviolet light and this feature makes the genetically engineered fish unique and attractive in the ornamental fish market. The fluorescent transgenic fish are also useful for the development of a biosensor system and as research models for embryonic studies such as cell lineage, cell migration, cell and nuclear transplantation etc.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1A-1I are photographs showing expression of CK (Figs. 1A-1C), MCK (Figs. 1D-1E), ARP (Figs. 1F-1G) and MLC2f (Figs. 1H-1I) mRNAs in zebrafish embryos as revealed by whole mount *in situ* hybridization (detailed description of the procedure can be found in Thisse et al., 1993). (Fig. 1A) A 28 hpf (hour postfertilization) embryo hybridized with a CK antisense riboprobe. (Fig. 1B) Enlargement of the mid-part of the embryo shown in Fig. 1A. (Fig. 1C) Cross-section of the embryo in Fig. 1A. (Fig. 1D) A 30 hpf embryo hybridized with an MCK antisense riboprobe. (Fig. 1E) Cross-section of the embryo in Fig 1D. (Fig. 1F) A 28 hpf embryo hybridized with an ARP antisense riboprobe. (Fig. 1G) Cross-section of the embryo in Fig. 1F. Arrows indicate the planes for cross-sections and the box in panel A indicates the enlarged region shown in panel B. (Fig. 1H) Side view of a 22-hpf embryo hybridized with the MLC2f probe. (Fig. 1I) Transverse

section through the trunk of a stained 24-hpf embryo. SC, spinal cord; N, notochord.

Fig. 2A is a digitized image showing distribution of CK, MCK and ARP mRNAs in adult tissues. Total RNAs were prepared from selected adult tissues as indicated at the top of each lane and analyzed by Northern blot hybridization (detailed description of the procedure can be found in Gong et al., 1992). Three identical blots were made from the same set of RNAs and hybridized with the CK, MCK and ARP probes, respectively.

Fig. 2B is a digitized image showing distribution of MLC2f mRNA in adult tissues. Total RNAs were prepared from selected adult tissues as indicated at the top of each lane and analyzed by Northern blot hybridization (detailed description of the procedure can be found in Gong et al., 1992). Two identical blots were made from the same set of RNAs and hybridized with the MLC2f probe and a ubiquitously expressed □-*actin* probe, respectively.

Fig. 3. is a schematic representation of the strategy of promoter cloning. Restriction enzyme digested genomic DNA was ligated with a short linker DNA which consists of Oligo 1 and Oligo 2. Nested PCR reactions were then performed: the first round PCR used linker specific primer L1 and gene specific primers G1, where G1 is CK1, MCK1, M1 or ARP1 in the described embodiments, and the second round linker specific primer L2 and gene specific primer G2, where G2 is CK2, MCK2, M2 or ARP2, respectively in the described embodiments.

Fig. 4 is a schematic map of the chimeric gene construct, pCK-EGFP. The 2.2 kb zebrafish DNA fragment comprising the CK promoter region is inserted into pEGFP-1 (Clonetech) at the EcoRI and BamHI site as indicated. In the resulting chimeric DNA construct, the EGFP gene is under control of the zebrafish CK promoter. Also shown is the kanamycin/neomycin resistance gene (Kan^r/Neo^r) in the backbone of the original pEGFP-1 plasmid. The total length of the recombinant plasmid pCK-EGFP is 6.4 kb.

Fig. 5 is a schematic map of the chimeric gene construct, pMCK-EGFP. The 1.5 kb zebrafish DNA fragment comprising the MCK promoter region is inserted into pEGFP-1 (Clonetech) at the EcoRI and BamHI site as indicated. In the resulting chimeric DNA construct, the EGFP gene is under control of the zebrafish MCK promoter. Also shown is the kanamycin/neomycin resistance gene (Kan^r/Neo^r) in the backbone of the original pEGFP-1 plasmid. The total length of the recombinant plasmid pMCK-EGFP is 5.7 kb.

Fig. 6 is a schematic map of the chimeric gene construct, pARP-EGFP. The 2.2 kb zebrafish DNA fragment comprising the ARP promoter/1st intron region is inserted into pEGFP-1 (Clonetech) at the EcoRI and BamHI site as indicated. In the resulting chimeric

DNA construct, the EGFP gene is under control of the zebrafish ARP promoter. Also shown is the kanamycin/neomycin resistance gene (Kan^r/Neo^r) in the backbone of the original pEGFP-1 plasmid. The total length of the recombinant plasmid pARP-EGFP is 6.4 kb.

Fig. 7 is a schematic map of the chimeric gene construct, pMLC2f-EGFP. The 2.0 kb zebrafish DNA fragment comprising the MLC2f promoter region is inserted into pEGFP-1 (Clonetech) at the HindIII and BamHI site as indicated. In the resulting chimeric DNA construct, the EGFP gene is under control of the zebrafish MLC2f promoter. Also shown is the kanamycin/neomycin resistance gene (Kan^r/Neo^r) in the backbone of the original pEGFP-1 plasmid. The total length of the recombinant plasmid pMLC2f-EGFP is 6.2 kb.

Fig. 8 is a photograph of a typical transgenic zebrafish fry (4 days old) with pCK-EGFP, which emits green fluorescence from skin epithelia under a blue light.

Fig. 9 is a photograph of a typical transgenic zebrafish fry (3 days old) with pMCK-EGFP, which emits green fluorescence from skeletal muscles under a blue light.

Fig. 10 is a photograph of a typical transgenic zebrafish fry (2 days old) with pARP-EGFP, which emits green fluorescence under a blue light from a variety of cell types such as skin epithelia, muscle cells, lens, neural tissues, notochord, circulating blood cells and yolk cells.

Figs. 11A-11B. Photographs of a typical transgenic zebrafish founder with pMLC2f-EGFP (Fig. 11A) and an F1 stable transgenic offspring (Fig. 11B). Both pictures were taken under an ultraviolet light (365 nm). The green fluorescence can be better observed under a blue light with an optimal wavelength of 488 nm.

Figs. 12A-12C. Examples of high, moderate and low expression of GFP in transiently transgenic embryos at 72 hpf. (Fig. 12A) High expression, GFP expression was detected in essentially 100% of the muscle fibers in the trunk. (Fig. 12B) Moderate expression, GFP expression was detected in several bundles of muscle fibers, usually in the mid-trunk region. (Fig. 12C) Low expression, GFP expression occurred in dispersed muscle fibers and the number of GFP positive fibers is usually less than 20 per embryo.

Fig. 13. Deletion analysis of the MLC2f promoter in transient transgenic zebrafish embryos. A series of 5' deletions of MLC2f-EGFP constructs containing –2011-bp (2-kb), –1338-bp, –873-bp, –283-bp, –77-bp and –3-bp of the MLC2f promoter were generated by unidirectional deletion using the double-stranded Nested Deletion Kit from Pharmacia

based on the manufacturer's instructional manual. Each construct was injected into approximately 100 embryos and GFP expression was monitored in the first 72 hours of embryonic development. The level of GFP expression was classified based on the examples shown in Figs. 12A-12C. Potential E-boxes and MEF2 binding sites, which are important for muscle-specific transcription (Schwarz et al., 1993; Olson et al., 1995), are indicated on the –2011-bp construct.

DETAILED DESCRIPTION OF THE INVENTION

Gene Constructs

To develop successful transgenic fish with a predictable pattern of transgene expression, the first step is to make a gene construct suitable for transgenic studies. The gene construct generally comprises three portions: a gene promoter, a structural gene and transcriptional termination signals. The gene promoter would determine where, when and under what conditions the structural gene is turned on. The structural gene contains protein coding portions that determine the protein to be synthesized and thus the biological function. The structural gene might also contain intron sequences which can affect mRNA stability or which might contain transcription regulatory elements. The transcription termination signals consist of two parts: a polyadenylation signal and a transcriptional termination signal after the polyadenylation signal. Both are important to terminate the transcription of the gene. Among the three portions, selection of a promoter is very important for successful transgenic study, and it is preferable to use a homologous promoter (homologous to the host fish) to ensure accurate gene activation in the transgenic host.

A promoter drives expression "predominantly" in a tissue if expression is at least 2-fold, preferably at least 5-fold higher in that tissue compared to a reference tissue. A promoter drives expression "specifically" in a tissue if the level of expression is at least 5-fold, preferably at least 10-fold higher, more preferably at least 50-fold higher in that tissue than in any other tissue.

Recombinant DNA Constructs

Recombinant DNA constructs comprising one or more of the DNA or RNA sequences described herein and an additional DNA and/or RNA sequence are also included within the scope of this invention. These recombinant DNA constructs usually have sequences which do not occur in nature or exist in a form that does not occur in nature or exist in association with other materials that do not occur in nature. The DNA and/or RNA sequences described as constructs or in vectors above are "operably linked" with other

DNA and/or RNA sequences. DNA regions are operably linked when they are functionally related to each other. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as part of a preprotein which participates in the secretion of the polypeptide; a promoter is operably linked to a coding sequence if it controls the transcription of the coding sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation. Generally, operably linked means contiguous (or in close proximity to) and, in the case of secretory leaders, contiguous and in reading phase.

The sequences of some of the DNAs, and the corresponding proteins encoded by the DNA, which are useful in the invention are set forth in the attached Sequence Listing.

The complete cytokeratin (CK) cDNA sequence is shown in SEQ ID NO:1, and its deduced amino acid sequence is shown in SEQ ID NO:2. The binding sites of the gene specific primers for promoter amplification, CK1 and CK2, are indicated. The extra nucleotides introduced into CK2 for generation of a restriction site are shown as a misc_feature in the primer sequence SEQ ID NO:11. A potential polyadenylation signal, AATAAA, is indicated in SEQ ID NO:1.

The complete muscle creatine kinase (MCK) cDNA sequence is shown in SEQ ID NO:3, and its deduced amino acid sequence is shown in SEQ ID NO:4. The binding sites of the gene specific primers for promoter amplification, MCK1 and MCK2, are indicated. The extra nucleotides introduced into MCK1 and MCK2 for generation of restriction sites are shown as a misc_feature in the primer sequences SEQ ID NOS:12 and 13, respectively. A potential polyadenylation signal, AATAAA, is indicated in SEQ ID NO:3.

The complete fast skeletal muscle isoform of myosin light chain 2 (MLC2f) cDNA sequence is shown in SEQ ID NO:20, and its deduced amino acid sequence is shown in SEQ ID NO:21. The binding sites of the gene-specific primers for promoter amplification, M1 and M2, are indicated. Two potential polyadenylation signals, AATAAA, are shown as a misc_feature in SEQ ID NO:20.

The complete acidic ribosomal protein P0 (ARP) cDNA sequence is shown in SEQ ID NO:5, and its deduced amino acid sequence is shown in SEQ ID NO:6. The binding sites of the gene specific primers for promoter amplification, ARP1 and ARP2, are indicated. The extra nucleotides introduced into ARP2 for generation of a restriction site are shown as a misc_feature in the primer sequence SEQ ID NO:15. A potential polyadenylation signal, AATAAA, is indicated in SEQ ID NO:5.

SEQ ID NO:7 shows the complete sequence of the CK promoter region. A putative

TATA box is shown, and the 3' nucleotides identical to the 5' CK cDNA sequence are shown as a misc_feature. The binding site of the second gene specific primer, CK2, is shown. The introduced BamHI site is indicated as a misc_feature in the primer sequence SEQ ID NO:11.

SEQ ID NO:8 shows the complete sequence of the MCK promoter region. A putative TATA box is shown, and the 3' nucleotides identical to the 5' MCK cDNA sequence are shown as a misc_feature in SEQ ID NO:8. The binding site of the second gene specific primer, MCK2, is shown. The introduced BamHI site is indicated as a misc_feature in the primer sequence SEQ ID NO:13.

SEQ ID NO:22 shows the complete sequence of the MLC2f promoter region. A putative TATA box is shown, and the 3' nucleotides identical to the 5' MLC2f cDNA sequence are shown as a misc_feature. The binding site of the second gene-specific primer, M2, is shown. Potential muscle-specific cis-elements, E-boxes and MEF2 binding sites, are also shown. The proximal 1-kb region of the MLC2f promoter was recently published (Xu et al., 1999).

SEQ ID NO:9 shows the complete sequence of the ARP promoter region including the first intron. The first intron is shown, and the 3' nucleotides identical to the 5' ARP cDNA sequence are shown as misc_features. No typical TATA box is found. The binding site of the second gene specific primer, ARP2, is shown. The introduced BamHI site is indicated as a misc_feature in the primer sequence SEQ ID NO:15.

Specifically Exemplified Polypeptides/DNA

The present invention contemplates use of DNA that codes for various polypeptides and other types of DNA to prepare the gene constructs of the present invention. DNA that codes for structural proteins, such as fluorescent peptides including GFP, EGFP, BFP, EBFP, YFP, EYFP, CFP, ECFP and enzymes (such as luciferase, ß-galactosidase, chloramphenicol acetyltransferase, etc.), and hormones (such as growth hormone etc.), are useful in the present invention. More particularly, the DNA may code for polypeptides comprising the sequences exemplified in SEQ ID NOS:2, 4, 6 and 21. The present invention also contemplates use of particular DNA sequences, including regulatory sequences, such as promoter sequences shown in SEQ ID NOS: 7, 8, 9 and 22 or portions thereof effective as promoters. Finally, the present invention also contemplates the use of additional DNA sequences, described generally herein or described in the references cited herein, for various purposes.

Chimeric Genes

The present invention also encompasses chimeric genes comprising a promoter described herein operatively linked to a heterologous gene. Thus, a chimeric gene can comprise a promoter of a zebrafish operatively linked to a zebrafish structural gene other than that normally found linked to the promoter in the genome. Alternatively, the promoter can be operatively linked to a gene that is exogenous to a zebrafish, as exemplified by the GFP and other genes specifically exemplified herein. Furthermore, a chimeric gene can comprise an exogenous promoter linked to any structural gene not normally linked to that promoter in the genome of an organism.

Variants of Specifically Exemplified Polypeptide

DNA that codes for variants of the specifically exemplified polypeptides are also encompassed by the present invention. Possible variants include allelic variants and corresponding polypeptides from other organisms, particularly other organisms of the same species, genus or family. The variants may have substantially the same characteristics as the natural polypeptides. The variant polypeptide will possess the primary property of concern for the polypeptide. For example, the polypeptide will possess one or more or all of the primary physical (e.g., solubility) and/or biological (e.g., enzymatic activity, physiologic activity or fluorescence excitation or emission spectrum) properties of the reference polypeptide. DNA of the structural genes of the present invention will encode a protein that produces a fluorescent or chemiluminescent light under conditions appropriate to the particular polypeptide in one or more tissues of a fish. Preferred tissues for expression are skin, muscle, eye and bone.

Substitutions, Additions and Deletions

As possible variants of the above specifically exemplified polypeptides, the polypeptide may have additional individual amino acids or amino acid sequences inserted into the polypeptide in the middle thereof and/or at the N-terminal and/or C-terminal ends thereof so long as the polypeptide possesses the desired physical and/or biological characteristics. Likewise, some of the amino acids or amino acid sequences may be deleted from the polypeptide so long as the polypeptide possesses the desired physical and/or biochemical characteristics. Amino acid substitutions may also be made in the sequences so long as the polypeptide possesses the desired physical and biochemical characteristics. DNA coding for these variants can be used to prepare gene constructs of the present invention.

Sequence Identity

The variants of polypeptides or polynucleotides contemplated herein should possess more than 75% sequence identity (sometimes referred to as homology), preferably more than 85% identity, most preferably more than 95% identity, even more preferably more than 98% identity to the naturally occurring and/or specifically exemplified sequences or fragments thereof described herein. To determine this homology, two sequences are aligned so as to obtain a maximum match using gaps and inserts.

Two sequences are said to be "identical" if the sequence of residues is the same when aligned for maximum correspondence as described below. The term "complementary" applies to nucleic acid sequences and is used herein to mean that the sequence is complementary to all or a portion of a reference polynucleotide sequence.

Optimal alignment of sequences for comparison can be conducted by the local homology algorithm of Smith and Waterman (1981), by the homology alignment method of Needleman and Wunsch (1970), by the search for similarity method of Pearson and Lippman (1988), or the like. Computer implementations of the above algorithms are known as part of the Genetics Computer Group (GCG) Wisconsin Genetics Software Package (GAP, BESTFIT, BLASTA, FASTA and TFASTA), 575 Science Drive, Madison, WI. These programs are preferably run using default values for all parameters.

"Percentage of sequence identity" is determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the sequence in the comparison window may comprise additions or deletions (*i.e.* "gaps") as compared to the reference sequence for optimal alignment of the two sequences being compared. The percentage identity is calculated by determining the number of positions at which the identical residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window and multiplying the result by 100 to yield the percentage of sequence identity. Total identity is then determined as the average identity over all of the windows that cover the complete query sequence.

Fragments of Polypeptide

Genes which code for fragments of the full length polypeptides such as proteolytic cleavage fragments which contain at least one, and preferably all, of the above-listed physical and/or biological properties are also encompassed by the present invention.

DNA and RNA

The invention encompasses DNA that codes for any one of the above-described polypeptides including, but not limited to, those shown in SEQ ID NOS:2, 4, 6 and 21 including fusion polypeptides, variants and fragments thereof. The sequence of certain particularly useful cDNAs which encode polypeptides are shown in SEQ ID NOS:1, 3, 5 and 20. The present invention also includes cDNA as well as genomic DNA containing or comprising the requisite nucleotide sequences as well as corresponding RNA and antisense sequences.

Cloned DNA within the scope of the invention also includes allelic variants of the specific sequences presented in the attached Sequence Listing. An "allelic variant" is a sequence that is a variant from that of the exemplified nucleotide sequence, but represents the same chromosomal locus in the organism. In addition to those which occur by normal genetic variation in a population and perhaps fixed in the population by standard breeding methods, allelic variants can be produced by genetic engineering methods. A preferred allelic variant is one that is found in a naturally occurring organism, including a laboratory strain. Allelic variants are either silent or expressed. A silent allele is one that does not affect the phenotype of the organism. An expressed allele results in a detectable change in the phenotype of the trait represented by the locus.

A nucleic acid sequence "encodes" or "codes for" a polypeptide if it directs the expression of the polypeptide referred to. The nucleic acid can be DNA or RNA. Unless otherwise specified, a nucleic acid sequence that encodes a polypeptide includes the transcribed strand, the hnRNA and the spliced RNA or the DNA representative of the mRNA. An "antisense" nucleic acid is one that is complementary to all or part of a strand representative of mRNA, including untranslated portions thereof.

Degenerate Sequences

In accordance with degeneracy of genetic code, it is possible to substitute at least one base of the base sequence of a gene by another kind of base without causing the amino acid sequence of the polypeptide produced from the gene to be changed. Hence, the DNA of the present invention may also have any base sequence that has been changed by substitution in accordance with degeneracy of genetic code.

DNA Modification

The DNA is readily modified by substitution, deletion or insertion of nucleotides, thereby resulting in novel DNA sequences encoding the polypeptide or its derivatives.

These modified sequences are used to produce mutant polypeptide and to directly express the polypeptide. Methods for saturating a particular DNA sequence with random mutations and also for making specific site-directed mutations are known in the art; see *e.g.* Sambrook et al. (1989).

Hybridizable Variants

The DNA molecules useful in accordance with the present invention can comprise a nucleotide sequence selected from the group consisting of SEQ ID NOS.:1, 3, 5, 7-20 and 22-24 or can comprise a nucleotide sequence that hybridizes to a DNA molecule comprising the nucleotide sequence of SEQ ID NOS.:1, 3, 5 or 20 under salt and temperature conditions providing stringency at least as high as that equivalent to 5x SSC and 42°C and that codes on expression for a polypeptide that has one or more or all of the above-described physical and/or biological properties. The present invention also includes polypeptides coded for by these hybridizable variants. The relationship of stringency to hybridization and wash conditions and other considerations of hybridization can be found in Chapters 11 and 12 of Sambrook et al (1989). The present invention also encompasses functional promoters which hybridize to SEQ ID NOS:7, 8, 9 or 22 under the above-described conditions. DNA molecules of the invention will preferably hybridize to reference sequences under more stringent conditions allowing the degree of mismatch represented by the degrees of sequence identity enumerated above. The present invention also encompasses functional primers or linker oligonucleotides set forth in SEQ ID NOS:10-19 and 23-24 or larger primers comprising these sequences, or sequences which hybridize with these sequences under the above-described conditions. The primers usually have a length of 10-50 nucleotides, preferably 15-35 nucleotides, more preferably 18-30 nucleotides.

Vectors

The invention is further directed to a replicable vector containing cDNA that codes for the polypeptide and that is capable of expressing the polypeptide.

The present invention is also directed to a vector comprising a replicable vector and a DNA sequence corresponding to the above described gene inserted into said vector. The vector may be an integrating or non-integrating vector depending on its intended use and is conveniently a plasmid.

Transformed Cells

The invention further relates to a transformed cell or microorganism containing cDNA or a vector which codes for the polypeptide or a fragment or variant thereof and that

is capable of expressing the polypeptide.

Expression Systems Using Vertebrate Cells

Interest has been great in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure. Examples of vertebrate host cell lines useful in the present invention preferably include cells from any of the fish described herein. Expression vectors for such cells ordinarily include (if necessary) an origin of replication, a promoter located upstream from the gene to be expressed, along with a ribosome-binding site, RNA splice site (if intron-containing genomic DNA is used or if an intron is necessary to optimize expression of a cDNA), a polyadenylation site, and a transcription termination sequence.

EXAMPLES

The following examples are provided by way of illustration only and not by way of limitation. Those of skill will readily recognize a variety of noncritical parameters which can be changed or modified to yield essentially similar results.

Example I: Isolation of skin-specific, muscle-specific and ubiquitously expressed zebrafish cDNA clones.

cDNA clones were isolated and sequenced as described by Gong et al. (1997). Basically, random cDNA clones were selected from zebrafish embryonic and adult cDNA libraries and each clone was partially sequenced by a single sequencing reaction. The partial sequences were then used to identify the sequenced clones for potential function and tissue specificity. Of the distinct clones identified by this approach, four of them were selected: for skin specificity (clone A39 encoding cytokeratin, CK), for muscle specificity (clone E146 encoding muscle creatine kinase, MCK), for skeletal muscle specificity (clone A113 encoding the fast skeletal muscle isoform of the myosin light chain 2, MLC2f) and for ubiquitous expression (clone A150 encoding acidic ribosomal protein P0, ARP), respectively.

The four cDNA clones were sequenced, and their complete cDNA sequences with deduced amino acid sequences are shown in SEQ ID NOS:1, 3, 5, and 20 respectively. A39 encodes a type II basic cytokeratin and its closest homolog in mammals is cytokeratin 8 (65-68% amino acid identity). E146 codes for the zebrafish MCK and its amino acid sequence shares ~87% identity with mammalian MCKs. A113 encodes the fast skeletal muscle isoform of the myosin light chain 2. The deduced amino acid sequence of this gene is highly homologous to other vertebrate fast skeletal muscle MLC2f proteins (over 80%

amino acid identity). The amino acid sequence of zebrafish ARP deduced from the A150 clone is 87-89% identical to those of mammalian ARPs.

To demonstrate their expression patterns, whole mount *in situ* hybridization (Thisse et al., 1993) was performed for developing embryos and Northern blot analyses (Gong et al., 1992) were carried out for selected adult tissues and for developing embryos.

As indicated by whole mount *in situ* hybridization, cytokeratin mRNA was specifically expressed in the embryonic surface (Figs. 1A-1C) and cross section of *in situ* hybridized embryos confirmed that the expression was only in skin epithelia (Fig. 1C). Ontogenetically, the cytokeratin mRNA appeared before 4 hours post-fertilization (hpf) and it is likely that the transcription of the cytokeratin gene starts at mid-blastula transition when the zygotic genome is activated. By *in situ* hybridization, a clear cytokeratin mRNA signal was detected in highly flattened cells of the superficial layer in blastula and the expression remained in the superficial layer which eventually developed into skin epithelia including the yolk sac. In adult tissues, cytokeratin mRNA was predominantly detected in the skin and also weakly in several other tissues including the eye, gill, intestine and muscle, but not in the liver and ovary (Fig. 2). Therefore, the cytokeratin mRNA is predominantly, if not specifically, expressed in skin cells.

MCK mRNA was first detected in the first few anterior somites in 10 somite stage embryos (14 hpf) and at later stages the expression is specifically in skeletal muscle (Fig. 1D) and in heart (data not shown). When the stained embryos are cross-sectioned, the MCK mRNA signal was found exclusively in the trunk skeletal muscles (Fig. 1E). In adult tissues, MCK mRNA was detected exclusively in the skeletal muscle (Fig. 2).

MLC2f mRNA was specifically expressed in fast skeletal muscle in developing zebrafish embryos (Figs.1H-1I). To examine the tissue distribution of MLC2f mRNA, total RNAs were prepared from several adult tissues including heart, brain, eyes, gills, intestine, liver, skeletal muscle, ovary, skin, and testis. MLC2f mRNA was only detected in the skeletal muscle by Northern analysis; while α-actin mRNA was detected ubiquitously in the same set of RNAs, confirming the validity of the assay (Fig. 2B).

ARP mRNA was expressed ubiquitously and it is presumably a maternal mRNA since it is present in the ovary as well as in embryos at one cell stage. In *in situ* hybridization experiments, an intense hybridization signal was detected in most tissues. An example of a hybridized embryo at 28 hpf is shown in Fig. 1F. In adults, ARP mRNA was abundantly expressed in all tissues examined except for the brain where a relatively weak signal was detected (Fig. 2A). These observations confirmed that the ARP mRNA is expressed ubiquitously.

Example II: Isolation of zebrafish gene promoters

Four zebrafish gene promoters were isolated by a linker-mediated PCR method as described by Liao *et al.*, (1997) and as exemplified by the diagrams in Fig. 3. The whole procedure includes the following steps: 1) designing of gene specific primers; 2) isolation of zebrafish genomic DNA; 3) digestion of genomic DNA by a restriction enzyme; 4) ligation of a short linker DNA to the digested genomic DNA; 5) PCR amplification of the promoter region; and 6) DNA sequencing to confirm the cloned DNA fragment. The following is the detailed description of these steps.

1. Designing of gene specific primers

Gene specific PCR primers were designed based on the 5' end of the four cDNA sequences and the regions used for designing the primers are shown in SEQ ID NOS: 1, 3, 5 and 20.

The two cytokeratin gene specific primers are:

CK1 (SEQ ID NO:10)

CK2 (SEQ ID NO:11), where the first six nucleotides are for creation of an EcoRI site to facilitate cloning.

The two muscle creatine kinase gene specific primers are:

MCK1 (SEQ ID NO:12), where the first five nucleotides are for creation of an EcoRI site to facilitate cloning.

MCK2 (SEQ ID NO:13), where the first three nucleotides are for creation of an EcoRI site to facilitate cloning.

The two fast skeletal muscle isoform of myosin light chain 2 gene specific primers are:

M1 (SEQ ID NO:23)

M2 (SEQ ID NO:24)

The two acidic ribosomal protein P0 gene specific primers are:

ARP1 (SEQ ID NO:14)

ARP2 (SEQ ID NO:15), where the first six nucleotides are for creation of an EcoRI site to facilitate cloning.

2. Isolation of zebrafish genomic DNA

Genomic DNA was isolated from a single individual fish by a standard method (Sambrook *et al.*, 1989). Generally, an adult fish was quickly frozen in liquid nitrogen and ground into powder. The ground tissue was then transferred to an extraction buffer (10 mM Tris, pH 8, 0.1 M EDTA, 20 μg/ml RNase A and 0.5% SDS) and incubated at 37°C for 1 hour. Proteinase K was added to a final concentration of 100 μg/ml and gently mixed until the mixture appeared viscous, followed by incubation at 50°C for 3 hours with periodical swirling. The genomic DNA was gently extracted three times by phenol equilibrated with Tris-HCl (pH 8), precipitated by adding 0.1 volume of 3 M NaOAc and 2.5 volumes of ethanol, and collected by swirling on a glass rod, then rinsed in 70% ethanol.

3. Digestion of genomic DNA by a restriction enzyme

Genomic DNA was digested with the selected restriction enzymes. Generally, 500 units of restriction enzyme were used to digest 50 μg of genomic DNA overnight at the optimal enzyme reaction temperature (usually at 37°C).

4. Ligation of a short linker DNA to the digested genomic DNA

The linker DNA was assembled by annealing equal moles of the two linker oligonucleotides, Oligo1 (SEQ ID NO:16) and Oligo 2 (SEQ ID NO:17). Oligo 2 was phosphorylated by T4 polynucleotide kinase prior to annealing. Restriction enzyme digested genomic DNA was filled-in or trimmed with T4 DNA polymerase, if necessary, and ligated with the linker DNA. Ligation was performed with 1 μg of digested genomic DNA and 0.5 μg of linker DNA in a 20 μl reaction containing 10 units of T4 DNA ligase at 4°C overnight.

5. PCR amplification of promoter region

PCR was performed with Advantage Tth Polymerase Mix (Clontech). The first round of PCR was performed using a linker specific primer L1 (SEQ ID NO:18) and a gene specific primer G1 (CK1, MCK1, M1 or ARP1). Each reaction (50 μl) contains 5 μl of 10x Tth PCR reaction buffer (1X= 15 mM KOAc, 40 mM Tris, pH 9.3), 2.2 μl of 25 mM $Mg(OAc)_2$, 5 μl of 2 mM dNTP, 1 μl of L1 (0.2 μg/μl), 1 μl of G1 (0.2 μg/μl) , 33.8

μl of H_2O, and 1 μl (50 ng) of linker ligated genomic DNA and 1 μl of 50x Tth polymerase mix (Clontech). The cycling conditions were as follows: 94°C/1 min, 35 cycles of 94°C/30 sec and 68°C/6 min, and finally 68°C/8 min. After the primary round of PCR was completed, the products were diluted 100 fold. One μl of diluted PCR product was used as template for the second round of PCR (nested PCR) with a second linker specific primer L2 (SEQ ID NO:19) and a second gene specific primer G2 (CK2, MCK2, M2 or ARP2), as described for the primary PCR but with the following modification: 94°C/1 min, 25 cycles of 94°C/30 sec and 68°C/6 min, and finally 68°C/8 min. Both the primary and secondary PCR products were analyzed on a 1% agarose gel.

6. DNA sequencing to confirm the cloned DNA fragment

PCR products were purified from the agarose gel following electrophoresis and cloned into a TA vector, pT7Blue™ (Novogen). DNA sequencing was performed by dideoxynucleotide chain termination method using a T7 Sequencing Kit purchased from Pharmacia. Complete sequences of these promoter regions were obtained by automatic sequencing using a dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer) and an ABI 377 automatic sequencing machine.

The isolated cytokeratin DNA fragment comprising the gene promoter is 2.2 kb. In the 3' proximal region immediately upstream of a portion identical to the 3' part of the CK cDNA sequence, there is a putative TATA box perfectly matching to a consensus TATA box sequence. The 164 bp of the 3' region is identical to the 5' UTR (untranslated region) of the cytokeratin cDNA. Thus, the isolated fragment was indeed derived from the same gene as the cytokeratin cDNA clone (SEQ ID NO:7). Similarly, a 1.5 kb 5' flanking region was isolated from the muscle creatine kinase gene, a putative TATA box was also found in its 3' proximal region and the 3' region is identical to the 5' portion of the MCK cDNA clone (SEQ ID NO:8). For MLC2f, a 2 kb region was isolated from the fast skeletal muscle isoform of myosin light chain 2 gene and sequenced completely. The promoter sequence for MLC2f is shown in SEQ ID NO:22. The sequence immediately upstream of the gene specific primer M2 is identical to the 5' UTR of the MLC2f cDNA clone; thus, the amplified DNA fragments are indeed derived from the MLC2f gene. A perfect TATA box was found 30 nucleotides upstream of the transcription start site, which was defined by a primer extension experiment based on Sambrook et al. (1989). In the 2-kb region comprising the promoter, six E-boxes (CANNTG) and six potential MEF2 binding sites [C/T)TA(T/A)4TA(A/G)] were found and are indicated in SEQ ID NO:22. Both of these cis-element classes are important for muscle specific gene transcription (Schwarz *et al.*, 1993; Olson *et al.*, 1995). A 2.2 kb fragment was amplified for the ARP gene. By

alignment of its sequence with the ARP cDNA clone, a 1.3 kb intron was found in the 5' UTR (SEQ ID NO:9). As a result, the isolated ARP promoter is within a DNA fragment about 0.8 kb long.

Example III: Generation of green fluorescent transgenic fish

The isolated zebrafish gene promoters were inserted into the plasmid pEGFP-1 (Clonetech), which contains an EGFP structural gene whose codons have been optimized according to preferable human codons. Three promoter fragments were inserted into pEGFP-1 at the EcoRI and BamHI site and the resulting recombinant plasmids were named pCK-EGFP (Fig. 4), pMCK-EGFP (Fig. 5), and pARP-EGFP, respectively (Fig. 6). The promoter fragment for the MLC2f gene was inserted into the Hind III and Bam HI sites of the plasmid pEGFP-1 and the resulting chimeric DNA construct, pMLC2f-EGFP, is diagramed in Fig. 7.

Linearized plasmid DNAs at a concentrations of 500 μg/ml (for pCK-EGFP and pMCK-EGFP) and 100 μg/ml (for pMLC2f-EGFP) in 0.1 M Tris-HCl (pH 7.6)/0.25% phenol red were injected into the cytoplasm of 1- or 2-cell stage embryos. Because of a high mortality rate, pARP-EGFP was injected at a lower concentration (50 μg/ml). Each embryo received 300-500 pl of DNA. The injected embryos were reared in autoclaved Holtfreter's solution (0.35% NaCl, 0.01% KCl and 0.01% $CaCl_2$) supplemented with 1 μg/ml of methylene blue. Expression of GFP was observed and photographed under a ZEISS Axiovert 25 fluorescence microscope.

When zebrafish embryos received pCK-EGFP, GFP expression started about 4 hours after injection, which corresponds to the stage of ~30% epiboly. About 55% of the injected embryos expressed GFP at this stage. The early expression was always in the superficial layer of cells, mimicking endogenous expression of the CK gene as observed by *in situ* hybridization. At later stages, in all GFP-expressing fish, GFP was found predominantly in skin epithelia. A typical pCK-EGFP transgenic zebrafish fry at 4 days old is shown in Fig. 8.

Under the MCK promoter, no GFP expression was observed in early embryos before muscle cells become differentiated. By 24 hpf, about 12% of surviving embryos expressed GFP strongly in muscle cells and these GFP-positive embryos remain GFP-positive after hatching. The GFP expression was always found in many bundles of muscle fibers, mainly in the mid-trunk region and no expression was ever found in other types of cells. A typical pMCK-EGFP transgenic zebrafish fry (3 days old) is shown in Fig. 9.

Expression of pARP-EGFP was first observed 4 hours after injection at the 30%

epiboly stage. The timing of expression is similar to that of pCK-EGFP-injected embryos. However, unlike the pCK-EGFP transgenic embryos, the GFP expression under the ARP promoter occurred not only in the superficial layer of cells but also in deep layers of cells. In some batches of injected embryos, almost 100% of the injected embryos expressed initially. At later stages when some embryonic cells become overtly differentiated, it was found that the GFP expression occurred essentially in all different types of cells such as skin epithelia, muscle cells, lens, neural tissues, notochord, circulating blood cells and yolk cells (Fig. 10).

Under the MLC2f promoter, nearly 60% of the embryos expressed GFP. The earliest GFP expression started in trunk skeletal muscles about 19 hours after injection, which corresponds to the stage of 20-somite. Later, the GFP expression also occurred in head skeletal muscles including eye muscles, jaw muscles, gill muscles etc.

Transgenic founder zebrafish containing pMLC2f-EGFP emit a strong green fluorescent light under a blue or ultraviolet light (Fig. 11A). When the transgenic founders were crossed with wild-type fish, transgenic offspring were obtained that also displayed strong green fluorescence (Fig. 11B). The level of GFP expression is so high in the transgenic founders and offspring that green fluorescence can be observed when the fish are exposed to sunlight.

To identify the DNA elements conferring the strong promoter activity in skeletal muscles, deletion analysis of the 2-kb DNA fragment comprising the promoter was performed. Several deletion constructs, which contain 5' deletions of the MLC2f promoter upstream of the EGFP gene, were injected into the zebrafish embryos and the transient expression of GFP in early embryos (19-72 hpf) was compared. To facilitate the quantitative analysis of GFP expression, we define the level of expression as follows (Figs. 12A-12C):

Strong expression: GFP expression was detected in essentially 100% muscle fibers in the trunk.

Moderate expression: GFP expression was detected in several bundles of muscle fibers, usually in the mid-trunk region.

Weak expression: GFP expression occurred in dispersed muscle fibers and the number of GFP positive fibers is usually less than 20 per embryo.

As summarized in Fig. 13, deletion up to –283 bp maintained the GFP expression in skeletal muscles in 100% of the expressing embryos; however, the level of GFP expression from these deletion constructs varies greatly. Strong expression drops from

23% to 0% from the 2-kb (-2011 bp) promoter to the –283-bp promoter. Thus, only two constructs (–2011 bp and –1338 bp) are capable of maintaining the high level of expression and the highest expression was obtained only with the 2-kb promoter, indicating the importance of the promoter region of –1338 bp to –2011 bp for conferring the highest promoter activity.

The expression of GFP using pMLC2f-EGFP is much higher than that obtained using the pMCK-EGFP that contains a 1.5 kb of zebrafish *MCK* promoter. By the same assay in transient transgenic zebrafish embryos, only about 12% of the embryos injected with pMCK-EGFP expressed GFP. Among the expressing embryos, no strong expression was observed, and 70% and 30% showed moderate and weak expression, respectively. In comparison, about 60% of the embryos injected with pMLC2f-EGFP expressed GFP and 23%, 37% and 40% showed strong, moderate and weak expression, respectively.

Example IV: Potential applications of fluorescent transgenic fish

The fluorescent transgenic fish have use as ornamental fish in the market. Stably transgenic lines can be developed by breeding a GFP transgenic individual with a wild type fish or another transgenic fish. By isolation of more zebrafish gene promoters, such as eye-specific, bone-specific, tail-specific etc., and/or by classical breeding of these transgenic zebrafish, more varieties of fluorescent transgenic zebrafish can be produced. Previously, we have reported isolation of over 200 distinct zebrafish cDNA clones homologous to known genes (Gong et al., 1997). These isolated clones code for proteins in a variety of tissues and some of them are inducible by heat-shock, heavy metals, or hormones such as estrogens. By using the method of PCR amplification using gene-specific primers designed from the nucleotide sequences of these cDNAs, and the linker-specific primers described herein, the promoters of the genes represented by the cDNAs of Gong et al. can be used in the present invention. Thus, other tissue-specific promoters, hormone-inducible promoters, heavy-metal inducible promoters and the like from zebrafish can be isolated and used to make fluorescent zebrafish (or other fish species) that express a GFP or variant thereof, in response to the relevant compound.

Multiple color fluorescent fish may be generated by the same technique as blue fluorescent protein (BFP) gene, yellow fluorescent protein (YFP) gene and cyan fluorescent protein (CFP) gene are available from Clonetech. For example, a transgenic fish with GFP under an eye-specific promoter, BFP under a skin-specific promoter, and YFP under a muscle-specific promoter will show the following multiple fluorescent colors: green eyes, blue skin and yellow muscle. By recombining different tissue specific promoters and fluorescent protein genes, more varieties of transgenic fish of different

fluorescent color patterns will be created. By expression of two or more different fluorescent proteins in the same tissue, an intermediate color may be created. For example, expression of both GFP and BFP under a skin-specific promoter, a dark-green skin color may be created.

By using a heavy metal- (such as cadmium, cobalt, chromium) inducible or hormone- (such as estrogen, androgen or other steroid hormone) inducible promoter, a biosensor system may be developed for monitoring environmental pollution and for evaluating water quality for human consumption and aquacultural uses. In such a biosensor system, the transgenic fish will glow with a green fluorescence (or other color depending on the fluorescence protein gene used) when pollutants such as heavy metals and estrogens (or their derivatives) reach a threshold concentration in an aquatic environment. Such a biosensor system has advantages over classical analytical methods because it is rapid, visualizable, and capable of identifying specific compounds directly in complex mixture found in an aquatic environment, and is portable or less instrument dependent. Moreover, the biosensor system also provides direct information on biotoxicity and it is biodegradable and regenerative.

Environmental monitoring of several substances can be accomplished by either creating one transgenic fish having genes encoding different colored fluorescent proteins driven by promoters responsive to each substance. Then the particular colors exhibited the fish in an environment can be observed. Alternatively, a number of fish can be transformed with individual vectors, then the fish can be combined into a population for monitoring an environment and the colors expressed by each fish observed.

In addition, the fluorescent transgenic fish should also be valuable in the market for scientific research tools because they can be used for embryonic studies such as tracing cell lineage and cell migration. Cells from transgenic fish expressing GFP can also be used as cellular and genetic markers in cell transplantation and nuclear transplantation experiments.

The chimeric gene constructs demonstrated successfully in zebrafish in the present invention should also be applicable to other fish species such as medaka, goldfish, carp including koi, loach, tilapia, glassfish, catfish, angel fish, discus, eel, tetra, goby, gourami, guppy, Xiphophorus (swordtail), hatchet fish, Molly fish, pangasius, etc. The promoters described herein can be used directly in these fish species. Alternatively, the homologous gene promoters from other fish species can be isolated by the method described in this invention. For example, the isolated and characterized zebrafish cDNA clones and promoters described in this invention can be used as molecular probes to screen for homologous promoters in other fish species by molecular hybridization or by PCR.

Alternatively, one can first isolate the zebrafish cDNA and promoters based on the sequences presented in SEQ ID NOS:1, 3, 5, 7, 8, 9, 20 and 22 or using data from other sequences of cDNAs disclosed by Gong et al. 1997, by PCR and then use the zebrafish gene fragments to obtain homologous genes from other fish species by the methods mentioned above.

In addition, a strong muscle-specific promoter such as MLC2f is valuable to direct a gene to be expressed in muscle tissues for generation of other beneficial transgenic fish. For example, transgenic expression of a growth hormone gene under the muscle-specific promoter may stimulate somatic growth of transgenic fish. Such DNA can be introduced either by microinjection, electroporation, or sperm carrier to generate germ-line transgenic fish, or by direct injection of naked DNA into skeletal muscles (Xu et al., 1999) or into other tissues or cavities, or by a biolistic method (gene bombardment or gene gun) (Gomez-Chiarri et al., 1996).

SEQUENCE LISTING

<110> GONG, Zhiyuan
LAM, Toong Jin
JU, Bensheng
XU, Yanfei
HE, Jiangyan
YAN, Tie

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His Gly Gly Tyr Lys Ala Thr Asp Lys His Lys Thr Asp Leu Asn Phe
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Glu Asn Leu Lys Gly Gly Asp Asp Leu Asp Pro Asn Tyr Phe Leu Ser
        115                 120                 125
Ser Arg Val Arg Thr Gly Arg Ser Ile Lys Gly Tyr Pro Leu Pro Pro
    130                 135                 140
His Asn Ser Arg Gly Glu Arg Arg Ala Val Glu Lys Leu Ser Val Glu
145                 150                 155                 160
Ala Leu Ser Ser Leu Asp Gly Glu Phe Lys Gly Lys Tyr Tyr Pro Leu
                165                 170                 175
Lys Ser Met Thr Asp Asp Glu Gln Glu Gln Leu Ile Ala Asp His Phe
            180                 185                 190
Leu Phe Asp Lys Pro Val Ser Pro Leu Leu Leu Ala Ala Gly Met Ala
        195                 200                 205
Arg Asp Trp Pro Asp Ala Arg Gly Ile Trp His Asn Glu Asn Lys Ala
    210                 215                 220
Phe Leu Val Trp Val Lys Gln Glu Asp His Leu Arg Val Ile Ser Met
225                 230                 235                 240
Gln Lys Gly Gly Asn Met Lys Glu Val Phe Lys Arg Phe Cys Val Gly
                245                 250                 255
Leu Gln Arg Ile Glu Glu Ile Phe Lys Lys His Asn His Gly Phe Met
            260                 265                 270
Trp Asn Glu His Leu Gly Phe Val Leu Thr Cys Pro Ser Asn Leu Gly
        275                 280                 285
Thr Gly Leu Arg Gly Gly Val His Val Lys Leu Pro Lys Leu Ser Thr
    290                 295                 300
His Ala Lys Phe Glu Glu Ile Leu Thr Arg Leu Arg Leu Gln Lys Arg
305                 310                 315                 320
Gly Thr Gly Gly Val Asp Thr Ala Ser Val Gly Gly Val Phe Asp Ile
                325                 330                 335
Ser Asn Ala Asp Arg Ile Gly Ser Ser Glu Val Glu Gln Val Gln Cys
            340                 345                 350
```

```
Val Val Asp Gly Val Lys Leu Met Val Glu Met Glu Lys Lys Leu Gly
        355                 360                 365

Glu Gly Gln Ser Ile Asp Ser Met Ile Pro Ala Gln Lys
    370                 375                 380
```

<210> 5
<211> 1104
<212> DNA
<213> Danio rerio

<220>
<221> CDS
<222> (75)..(1034)

<220>
<221> primer_bind
<222> (45)..(64)
<223> ARP2

<220>
<221> primer_bind
<222> (87)..(112)
<223> ARP1

<220>
<221> polyA_signal
<222> (1069)..(1074)

<400> 5

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cgcgtcccta ccgtgagatt ttacaacctt gtctttaaac cggctgttca ccgatccttg 60

gaagcactgc aaag atg ccc agg gaa gac agg gcc acg tgg aag tcc aac   110
                Met Pro Arg Glu Asp Arg Ala Thr Trp Lys Ser Asn
                  1               5                  10

tat ttt ctg aaa atc atc caa ctg ctg gat gac ttc ccc aag tgt ttc   158
Tyr Phe Leu Lys Ile Ile Gln Leu Leu Asp Asp Phe Pro Lys Cys Phe
         15                  20                  25

atc gtg ggc gca gac aat gtc ggc tcc aag cag atg cag acc atc cgt   206
Ile Val Gly Ala Asp Asn Val Gly Ser Lys Gln Met Gln Thr Ile Arg
     30                  35                  40

ctg tcc ctg cgg ggc aag gcc gtc gtg ctc atg ggg aaa aac acc atg   254
Leu Ser Leu Arg Gly Lys Ala Val Val Leu Met Gly Lys Asn Thr Met
 45                  50                  55                  60

atg agg aag gcc att cgt ggc cac ctg gaa aac aac cca gct ctg gag   302
Met Arg Lys Ala Ile Arg Gly His Leu Glu Asn Asn Pro Ala Leu Glu
                 65                  70                  75

agg ctg ctt ccc cac atc cgc ggg aac gtg ggc ttc gtc ttc acc aag   350
Arg Leu Leu Pro His Ile Arg Gly Asn Val Gly Phe Val Phe Thr Lys
             80                  85                  90

gag gat ctg act gag gtc cga gac ctg ctg ctg gca aac aaa gtg ccc   398
Glu Asp Leu Thr Glu Val Arg Asp Leu Leu Leu Ala Asn Lys Val Pro
         95                  100                 105

gct gct gcc cgt gct ggt gcc atc gcc ccc tgt gag gtg act gtg ccg   446
Ala Ala Ala Arg Ala Gly Ala Ile Ala Pro Cys Glu Val Thr Val Pro
    110                 115                 120

gcc cag aac acc ggg ctc ggt cct gag aag acc tct ttc ttc cag gct   494
```

```
Ala Gln Asn Thr Gly Leu Gly Pro Glu Lys Thr Ser Phe Phe Gln Ala
125                 130                 135                 140

ttg gga atc acc acc aag atc tcc aga gga acc att gaa atc ttg agt   542
Leu Gly Ile Thr Thr Lys Ile Ser Arg Gly Thr Ile Glu Ile Leu Ser
    —           145                 150                 155

gac gtt cag ctt atc aaa cct gga gac aag gtg ggc gcc agc gag gcc   590
Asp Val Gln Leu Ile Lys Pro Gly Asp Lys Val Gly Ala Ser Glu Ala
            160                 165                 170

acg ctg ctg aac atg ctg aac atg ctg aac atc tcg ccc ttc tcc tac   638
Thr Leu Leu Asn Met Leu Asn Met Leu Asn Ile Ser Pro Phe Ser Tyr
        175                 180                 185

ggg ctg atc atc cag cag gtg tat gat aac ggc agt gtc tac agc ccc   686
Gly Leu Ile Ile Gln Gln Val Tyr Asp Asn Gly Ser Val Tyr Ser Pro
    190                 195                 200

gag gtg ctg gac atc act gag gac gcc ctg cac aag agg ttc ctg aag   734
Glu Val Leu Asp Ile Thr Glu Asp Ala Leu His Lys Arg Phe Leu Lys
205                 210                 215                 220

ggt gtg agg aac atc gcc agt gtg tgt ctg cag atc ggc tac cca act   782
Gly Val Arg Asn Ile Ala Ser Val Cys Leu Gln Ile Gly Tyr Pro Thr
                225                 230                 235

ctt gct tcc atc cct cac act atc atc aat gga tac aag agg gtc ctg   830
Leu Ala Ser Ile Pro His Thr Ile Ile Asn Gly Tyr Lys Arg Val Leu
            240                 245                 250

gct gtc act gtc gaa aca gac tac aca ttc ccc ttg gct gag aag gtg   878
Ala Val Thr Val Glu Thr Asp Tyr Thr Phe Pro Leu Ala Glu Lys Val
        255                 260                 265

aag gcc tac ctg gct gat ccc acc gct ttc gct gtt gca gcc cct gtt   926
Lys Ala Tyr Leu Ala Asp Pro Thr Ala Phe Ala Val Ala Ala Pro Val
    270                 275                 280

gcg gca gct aca gag cag aaa tcc gct gct cct gcg gct aaa gag gag   974
Ala Ala Ala Thr Glu Gln Lys Ser Ala Ala Pro Ala Ala Lys Glu Glu
285                 290                 295                 300

gca ccc aag gag gat tct gag gag tct gat gaa gac atg ggc ttc ggc   1022
Ala Pro Lys Glu Asp Ser Glu Glu Ser Asp Glu Asp Met Gly Phe Gly
                305                 310                 315

ctg ttt gat taa accagacacc gaatatccat gtctgtttaa catcaataaa       1074
Leu Phe Asp
            320

acatctggaa acaaaaaaaa aaaaaaaaaa                                  1104


<210> 6
<211> 319
<212> PRT
<213> Danio rerio

<400> 6
Met Pro Arg Glu Asp Arg Ala Thr Trp Lys Ser Asn Tyr Phe Leu Lys
  1               5                  10                  15

Ile Ile Gln Leu Leu Asp Asp Phe Pro Lys Cys Phe Ile Val Gly Ala
             20                  25                  30
```

```
Asp Asn Val Gly Ser Lys Gln Met Gln Thr Ile Arg Leu Ser Leu Arg
         35                  40                  45

Gly Lys Ala Val Val Leu Met Gly Lys Asn Thr Met Met Arg Lys Ala
     50                  55                  60

Ile Arg Gly His Leu Glu Asn Asn Pro Ala Leu Glu Arg Leu Leu Pro
 65                  70                  75                  80

His Ile Arg Gly Asn Val Gly Phe Val Phe Thr Lys Glu Asp Leu Thr
                 85                  90                  95

Glu Val Arg Asp Leu Leu Leu Ala Asn Lys Val Pro Ala Ala Ala Arg
            100                 105                 110

Ala Gly Ala Ile Ala Pro Cys Glu Val Thr Val Pro Ala Gln Asn Thr
        115                 120                 125

Gly Leu Gly Pro Glu Lys Thr Ser Phe Phe Gln Ala Leu Gly Ile Thr
    130                 135                 140

Thr Lys Ile Ser Arg Gly Thr Ile Glu Ile Leu Ser Asp Val Gln Leu
145                 150                 155                 160

Ile Lys Pro Gly Asp Lys Val Gly Ala Ser Glu Ala Thr Leu Leu Asn
                165                 170                 175

Met Leu Asn Met Leu Asn Ile Ser Pro Phe Ser Tyr Gly Leu Ile Ile
            180                 185                 190

Gln Gln Val Tyr Asp Asn Gly Ser Val Tyr Ser Pro Glu Val Leu Asp
        195                 200                 205

Ile Thr Glu Asp Ala Leu His Lys Arg Phe Leu Lys Gly Val Arg Asn
    210                 215                 220

Ile Ala Ser Val Cys Leu Gln Ile Gly Tyr Pro Thr Leu Ala Ser Ile
225                 230                 235                 240

Pro His Thr Ile Ile Asn Gly Tyr Lys Arg Val Leu Ala Val Thr Val
                245                 250                 255

Glu Thr Asp Tyr Thr Phe Pro Leu Ala Glu Lys Val Lys Ala Tyr Leu
            260                 265                 270

Ala Asp Pro Thr Ala Phe Ala Val Ala Ala Pro Val Ala Ala Ala Thr
        275                 280                 285

Glu Gln Lys Ser Ala Ala Pro Ala Ala Lys Glu Glu Ala Pro Lys Glu
    290                 295                 300

Asp Ser Glu Glu Ser Asp Glu Asp Met Gly Phe Gly Leu Phe Asp
305                 310                 315


<210> 7
<211> 2241
<212> DNA
<213> Danio rerio

<220>
<221> TATA_signal
<222> (2103)..(2108)
```

<220>
<221> primer_bind
<222> (2221)..(2241)
<223> CK2

<220>
<221> misc_feature
<222> (2142)..(2235)
<223> Identical to the 5' CK cDNA

<400> 7
ccttcccttc tacttttgac gtccttttaa gagcttgtgc atgaaagcag atttggagct 60

gattactcat ctcaaacacc catacaaagg gatgattgcc gtaccatgat ctcacacctt 120

tcacacctgg tttatactat gatagttgta gacgattgcg taatgctatt aaatgcccat 180

cagtgctggc tgtgacaccc aactgctgcc atttcgtgtt gacttgcacg agaaatgaga 240

aattgtctga ctatgcaggg tgtctatgcg tgggaacatt tatcagtggt cattaaatac 300

tatagtttac agttagacca aagtgtgctg tatttttgtg ttagcttagc tgcagttttt 360

gtgtgtgaag taacaaatga caaatactca aactattgta attaagtagt ttttctcaga 420

aattgtaatt tactaagtag tttaaaaatg tgtactttta ctttcccttg agtacatttt 480

tagtgcagtg ttggtacttt tatttcactt ccttccttca acctgcagtc actactttat 540

ttattcttgt ctatgtggat tagacaaatc agtcctgtga ttcctgtcca atcaaattgc 600

acatagaagg taaatcacat cataatgaac taccttaaga catgggccat ttataattgc 660

agcaaactgt ttgccagcat taaaagaaga tgtcaaaaat atttacacgc attaacccag 720

agactgctta gatgcatgtc actgatgaga agatgatgga tgtttactgt atgatgaccg 780

aaataacttt aaacgcacac aagacggcac aagacgtcaa catggcgtta ggttgacgtt 840

gtaccccaac gcagtgggga cgttgcattt tgtttagaaa tgaaaattag gttgacgtca 900

gaactcaacg tcaggtcgat gtcaatgttc aacatccaat ctaaaatcat atatcaatgt 960

ctaatgatgt tacagcttga tgttatgcgg atgttacccc tatgacgtct atcagacgtt 1020

ggattatggt tgccatacct gatgaataaa tgtcattatt tgacgttggt ttaagatgtt 1080

ggttcgacat tggattttgg tcgctttcca acacaaccta aatccaccaa atattaactt 1140

cctatgacat cgttattgga cgtcaaaata acaatatcct tagatgctgg ctagactttg 1200

aatttaggtc accacaacct atatttaacc taatattaac atcttatgat gttgtgtgcc 1260

tgctgggcaa taactaaatg cactacagaa tgttacgttt acacacatgt aaattacatg 1320

taaatgcatc agcttttcac agcataatac tcactactta ctactcttga gtacttttaa 1380

aaaagctact tttcactcat actttgagta atatttacaa ctgatacttt tactcgcact 1440

acatttttag gcatgtattg atatttttac tatgattttt cagtactctt tccactactg 1500

cagccctccc catacataat cgtatgttta cacatatggt ggagtttaga gccataatct 1560

acattagctt tgttagccgc tagcattact gtgcagaatt gtgtgtgtgc acattttcca 1620

atatcaatac agaaggaaac tgtgttccct gttcccttgt aaatctcaac aatgcaactg 1680

ttcagctcag ggggaaaaat gccctgccag atccaaacgg ctggcaaaag tgaatggaaa 1740

aaagcctttc attaatgtga aagttgctgc gcgccccacc cagataaaaa gagcagaggt 1800

taacatgctc tctacggctg tccagccaac cagatactga ggcagaaaca cacccgctgg 1860

cagatggtga gagctacact gtcttttcca gagtttctac tggaatgcct gtcctcaagt 1920

ctcaagcctc tccttgcatt ctctcattcc acctggggca aagccccagg ctgggtgtga 1980

caacatttat cttaccactt tctctctgta cctgtctaac aggtagggtg tgtgtgagag 2040

tgcgtatgtg tgcaagtgcg tgtgtgtgtg agagcagtca gctccaccct ctcaagagtg 2100

tgtataaaat tggtcagcca gctgctgaga gacacgcaga gggactttga ctctcctttg 2160

tgagcaacct cctccactca ctcctctctc agagagcact ctcgtacctc cttctcagca 2220

actcaaagac acaggatccg g 2241

<210> 8
<211> 1456
<212> DNA
<213> Danio rerio

<220>
<221> TATA_signal
<222> (1389)..(1394)

<220>
<221> primer_bind
<222> (1433)..(1456)
<223> MCK2

<220>
<221> misc_feature
<222> (1428)..(1453)
<223> Identical to the 5' MCK cDNA

<400> 8
gaattgcaaa gtcagagtaa taaaatgaaa ccaaaaaaca tttttaaata tacttgtctc 60

tgtggcttaa tcttggctga tgtgtgtgtg tgtgtgtgtg tacttgacag ctgctagtga 120

gcatgtgcac catgacaggc ctgttattca cacttggtgc catgttggag actgttcggc 180

cagctatagt tttcttcaca gagtcctggg tcacctaatg tcacaaggaa gaaacatgtt 240

acatgttaaa atgtgacatt caaattgtag tgcattactt aacgaaacgc attacacaag 300

ttacagctta aaagattgct agacagaaaa accagggagg ggttttccca taatatccag 360

tgagactcta ggagcgggaa cactaacagg cctccctgag tgagaacatt gcatgtgcgc 420

gtgacagaaa accagagatg gaaatacctt cttttgaatt gcataattgc ttaaaagaag 480

acacaacagg gatagttcac ccaaaaaaca gaccattctt tttttctgtt gaacaaaaat 540

taagatattt tgaagaatgc ttaccgaata acttccatat ttggaaacta attacagtga 600

aagtcaatgg gtcttccagc atttttttcaa tataccttac tttgagttca aaagaaaaac 660

acatctcaaa taggtttgag gttgaataaa catttttcat tttggggtgg actatcccta 720

attatttgac acttaagatt tatagtaaat cattttatag actttctccc cttattaaac 780

atggttgaat ttatcttcat gtttatgtct gggttgtgct tttttgaaaa gatttccctg 840

tcaaatgttt ttgtgtatgg ttggcgcaca atagactgaa ctggcctatc acacagactt 900

tcataacaac tccagttgat gccctttcac cctcagtgta taaatatggc gtctgacatg 960

agcagattaa acacgacact gcaacaactt tacctgtaaa aatacaaatt gagtttgcac 1020

ccagaatcat gtggtgaacg aagcctacca agagattttt gaaagccatc ggcctgacac 1080

gcgcacttct gatatctgtg gtatgtttgg caaaagtgct gctcagcctt tttagcatgg 1140

cagatcctcc acatcccatc acccctcctt caacctattc cctcctggaa agctatgtat 1200

ggggcgggaa gtgtaaatgg atatgggaag gaaggggggc accacccaca gctgccacct 1260

catctaggat gcctggggcc taaattgaag cctttcttac actaaacagg gcataagaga 1320

ccagcgccag ccaatcataa ttcagtgagc tctaaaatgg gccagccaat ggctgcaggg 1380

gctagaggta tatatatcca aatcaaactc ttcttgcttg ggtgacccct atttcggctt 1440

ggtgaacagg atccgg 1456

<210> 9
<211> 2205
<212> DNA
<213> Danio rerio

<220>
<221> primer_bind
<222> (2179)..(2205)
<223> ARP2

<220>
<221> misc_feature
<222> (2153)..(2199)
<223> Identical to the 5' ARP cDNA

<220>
<221> intron
<222> (792)..(2152)

<220>
<221> misc_feature
<222> (775)..(791)
<223> Identical to the 5' ARP cDNA

<400> 9
atctgtatta agaaacactt aaaatatata tgcgttacga attaaaaaca aaacacgatc 60

attttaattt gtgttgtata attttacatt ttgtaagtat tatttttata aaaaatatat 120

agaaataata caaatttgtt tacagtattc ttagttattg caataaacga attttatata 180

gaaagagaaa gagttttatt ataagatgtt caatttaaaa aatggcagaa aatagaaaaa 240

tgattgtcaa gatgataaaa gtcagtttag acaaaaaaat aagatgaaaa acatcaaaat 300

agataataaa gtgacttttt tgggcggacc aaatttccct attaatggtc aattcattaa 360

aatacattca ttaaaataaa ggtattgcga tgaatttaga tgcacagtga ttttggttct 420

gtgcagattt ttggctgttg ttagaaggga tacatctgcg gccgaaagtt aacgggaact 480

atttacattc tttgctatta aattatccat tatttgtatt ttattacccc aaccgtaaac 540

tcaaccctca cagtaatgta aaaatattat ttattgtttt atagcgtcac agaatgatgc 600

tatattgacc gcagctgtat cctttctaag tgcgactgta caaatacgca ctgaccgtga 660

cagacacgtg cattgaccaa tcagcgcaca gatacgcatt ttccgcgcga ttctgattgg 720

atgatcgact gatactaata ttgtgccgct tcctttcgcg gcctctttct ttcacgcgtc 780

cctaccgtga ggtaaggctg acgccgctct tgtggcggtt tcttaaaatg tgttaataaa 840

taacatcata agaggtcacg agaaggtcta cgtgtgttta atatcagcgg cggttattat 900

tatgcgttta aagcttgtgt aatgattttt acagtaaaag ttagcactag cctgttagca 960

caggcctcgt gcgccatgtg tgacgcgacg ttttaatagc atcttatttg attttgatga 1020

tccgattctg atattaatca tatttatgcg taaaatgtgt gatgggtctg ctagtggaca 1080

ttacatgcta gtacttgtgc tagtcggtcg atccacattg agatgttgcg ctatttgcca 1140

ttttaaaacc agttactctc attttagtga aatattctta agccactaag ttaaaatttg 1200

tcaatcacat ataattgtgt ttatgtttta tttgagtcat cataccaggt aatagtttta 1260

tttatattag tatgtacaat ttggcataaa ctgccttcgg ttttgattga catctacttt 1320

gtaaagttaa tcttaaaggg gtaaaggctc acccaaaaga caattcaccg tcaagtgttt 1380

tcaaatctta tgagtttctt aatgaacatg gtatgttttg gagaaaactg gaaaccaact 1440

accataatac aaatacagga aaaatatact atagaagtcg atggttacag gttttctgca 1500

ttcaaaatat ctacacaagt gtttaatgga aggaactcaa gtgatttgaa aagttaaggg 1560

tgcataaatc agttttcatt tgggtgagct gtctctaaac atttgattta gacacctcag 1620

gcagtggtca ccaagcttgt tcctgaaggg ccagtgtcct acagatttta gctccaaccc 1680

taattaaaca cacctgaaca agctaatcaa ggtcttacta ggtatgtttg aaacatccag 1740

gcaggtgtgt tgatgcaaga tagagctaaa ccctgcaggg acaatggccc aacaggattg 1800

gtgacccctg cctcaagcca tcacaaatgc attatggtat taagaaatgt gcaggttcag 1860

ttatggacag gctgttgcag tgcttgttcg tcgttcccac tgcacaaatg aacatgattc 1920

cttctatccc tgtctgtctg catctcatga cttgcaggga cgctggtctc agacacgttt 1980

atagcagtaa atcaaataca atagtgctct gattatcttt aaatatttga aagcttataa 2040

taggcaacca aattacctgg aaacagttta caaacagtaa ttcatatttt gtcatttaat 2100

aagatgcaca caaggcaggt gtaaaagtat tgcttgtgtt tgtaatcctc agattttaca 2160

accttgtctt taaaccggct gttcaccgat ccttggaagg gatcc 2205

<210> 10
<211> 24

<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Cytokeratin
— gene specific primer

<400> 10
cgctggagta agagatagac ctgg 24

<210> 11
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Cytokeratin
gene specific primer

<220>
<221> misc_feature
<222> (1)..(6)
<223> Introduced for restriction site

<220>
<221> misc_feature
<222> (3)..(8)
<223> BamHI site

<400> 11
ccggatcctg tgtctttgag ttgctg 26

<210> 12
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Muscle
creatine kinase gene specific primer

<220>
<221> misc_feature
<222> (3)..(8)
<223> BamHI site

<400> 12
ccggatcctt gggatcagat cctg 24

<210> 13
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Muscle
creatine kinase gene specific primer

<220>
<221> misc_feature
<222> (1)..(3)
<223> Introduced for restriction site

<220>
<221> misc_feature

<222> (3)..(8)
<223> BamHI site

<400> 13
ccggatcctg ttcaccaagc cgaa 24

<210> 14
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Acidic ribosomal protein PO gene specific primer

<400> 14
tagttggact tccacgtgcc ctgtc 25

<210> 15
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Acidic ribosomal protein PO gene specific primer

<220>
<221> misc_feature
<222> (1)..(7)
<223> Introduced for restriction site

<220>
<221> misc_feature
<222> (1)..(6)
<223> BamHI site

<400> 15
ggatcccttc caaggatcgg tgaaca 26

<210> 16
<211> 51
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Oligonucleotide for linker used in linker-mediated PCR

<400> 16
gttcatcttt acaagctagc gctgaacaat gctgtggaca agcttgaatt c 51

<210> 17
<211> 10

<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Oligonucleotide for linker used in linker-mediated PCR

<220>

<223> n is a dideoxycytidine

<400> 17
gaattcaagn 10

<210> 18
<211> 21

<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: linker
specific primer

<400> 18
gttcatcttt acaagctagc g 21

<210> 19
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: linker
specific primer

<400> 19
tcctgaacaa tgctgtggac 20

<210> 20
<211> 1392
<212> DNA
<213> Danio rerio

<220>
<221> CDS
<222> (42)..(551)

<220>
<221> primer_bind
<222> (6)..(28)
<223> M2

<220>
<221> primer_bind
<222> (23)..(45)
<223> M1

<220>
<221> polyA_signal
<222> (797)..(802)

<220>
<221> polyA_signal
<222> (1351)..(1357)

<400> 20
ctcttcttga tcttcttaga cttcacacat accgtctcga c atg gca ccc aag aag 56

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                                                    Met Ala Pro Lys Lys
                                                      1               5

gcc aag agg agg gca gca gga gga gag ggt tcc tcc aac gtc ttc tcc   104
Ala Lys Arg Arg Ala Ala Gly Gly Glu Gly Ser Ser Asn Val Phe Ser
    —           10                  15                  20

atg ttt gag cag agc cag att cag gag tac aaa gag gct ttc aca atc   152
Met Phe Glu Gln Ser Gln Ile Gln Glu Tyr Lys Glu Ala Phe Thr Ile
            25                  30                  35

att gac cag aac aga gac ggt atc atc agc aaa gac gac ctt agg gac   200
Ile Asp Gln Asn Arg Asp Gly Ile Ile Ser Lys Asp Asp Leu Arg Asp
        40                  45                  50

gtg ttg gcc tca atg ggc cag ctg aat gtg aag aat gag gag ctg gag   248

Val Leu Ala Ser Met Gly Gln Leu Asn Val Lys Asn Glu Glu Leu Glu
    55                  60                  65

gcc atg atc aag gaa gcc agc ggc cca atc aac ttc acc gtt ttc ctc   296
Ala Met Ile Lys Glu Ala Ser Gly Pro Ile Asn Phe Thr Val Phe Leu
70                  75                  80                  85

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Val Ser Ala Phe Lys Val Leu Asp Pro Glu Gly Thr Gly Ser Ile Lys
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Lys Glu Phe Leu Glu Glu Leu Leu Thr Thr Gln Cys Asp Arg Phe Thr
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Pro Glu Asp Val Ile Val Ser Ala Phe Lys Val Leu Asp Pro Glu Gly
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The following references, referred to in this application, are hereby incorporated by reference in their entirety.

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CLAIMS

1. A zebrafish cytokeratin gene promoter which is capable of directing a structural gene to be predominantly expressed in skin epithelia when it is inserted in front of the structural gene and introduced into fish embryos.

2. A zebrafish muscle creatine kinase gene promoter which is capable of directing a structural gene to be specifically expressed in muscles when it is inserted in front of the structural gene and introduced into fish embryos.

3. A zebrafish fast skeletal muscle isoform of myosin light chain 2 gene promoter which is capable of directing a structural gene to be predominantly expressed in skeletal muscles when it is inserted in front of the structural gene and introduced into fish embryos.

4. A zebrafish acidic ribosomal protein P0 gene promoter which is capable of directing a structural gene to be expressed ubiquitously in all tissues when it is inserted in front of the structural gene and introduced into fish embryos.

5. A recombinant DNA molecule comprising a structural gene and the promoter of claim 1, 2, 3 or 4 arranged upstream of said structural gene.

6. A chimeric gene comprising the promoter of claim 1, 2, 3 or 4, operatively linked to DNA encoding a protein selected from the group consisting of GFP, modified GFP, EGFP, BFP, EBFP, YFP, EYFP, CFP, ECFP, luciferase, ß-galactosidase, chloramphenicol acetyltransferase, and growth hormone.

7. A transgenic fish comprising a chimeric gene comprising the promoter of claim 1, 2, 3 or 4.

8. The transgenic fish of claim 7, which contains said promoter in germ cells and/or in somatic cells and which is capable of breeding with either a said transgenic fish or a non-transgenic fish to produce viable and fertile transgenic progeny.

9. The transgenic fish of claim 7, and progeny of said fish that emits green fluorescence when the whole fish is exposed to a blue or ultraviolet light.

10. A transgenic fish comprising a DNA that encodes a fluorescent protein under control of a promoter that causes said DNA (1) to be expressed in predominately

skin epithelia, (2) to be specifically expressed in muscles, (3) to be predominantly expressed in skeletal muscles, or (4) to be expressed ubiquitously in all tissues.

11. The transgenic fish of claim 8, wherein said fluorescent protein is expressed a level sufficient that said fish fluoresces upon exposure to sunlight or daylight.

12. The transgenic fish of claim 11, wherein said high expression is induced by exposure of said fish to a steroid compound or to a heavy metal.

13. The transgenic fish of claim 10, wherein said promoter is a promoter which naturally occurs in the genome of a fish of the same species as the transgenic fish.

14. A recombinant DNA vector comprising a promoter DNA that hybridizes under stringent conditions to a polynucleotide of any one of SEQ ID NOS:7, 8, 9, or 22, operatively linked to a structural gene encoding a fluorescent or chemiluminescent protein.

15. A cell transformed with the vector of claim 14.

16. A transgenic fish comprising a chimeric gene in turn comprising a promoter DNA that hybridizes under stringent conditions to a polynucleotide of any one of SEQ ID NOS:7, 8, 9, or 22, operatively linked to a structural gene encoding a fluorescent or a chemiluminescent protein.

17. A method for sensing a steroid hormone or a steroid hormone derivative in a water sample comprising:

(a) contacting a fish expressing a fluorescent or chemiluminescent protein under control of an estrogen- or other steroid hormone-inducible promoter with a sample of water; and

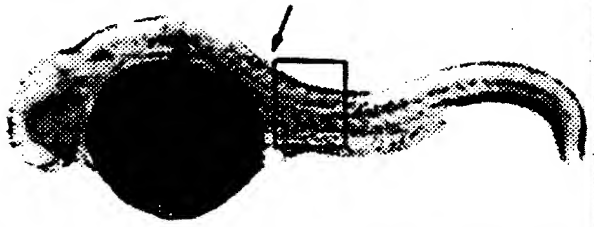
(b) measuring the amount of fluorescent or chemiluminescent light from said fish.

18. A method for sensing heavy metals, such as zinc, copper, cadmium, mecury etc., in a water sample comprising:

(a) contacting a fish expressing a fluorescent or chemiluminescent protein under control of a heavy metal-inducible promoter with a sample of water; and

(b) measuring the amount of fluorescent or chemiluminescent light from said fish.

Fig. 1A



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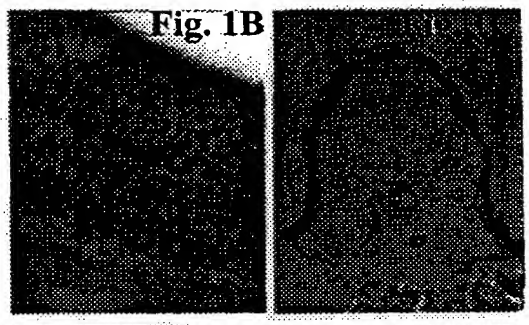

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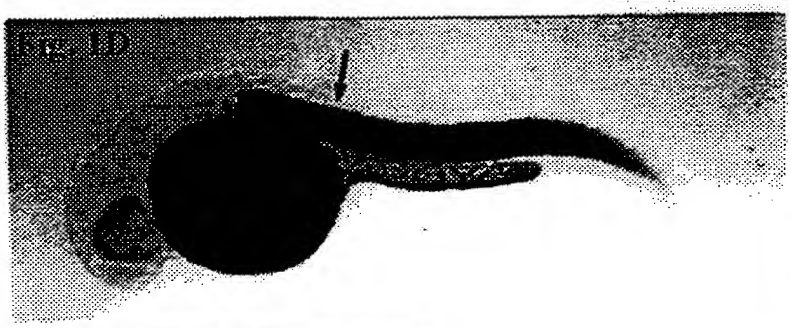

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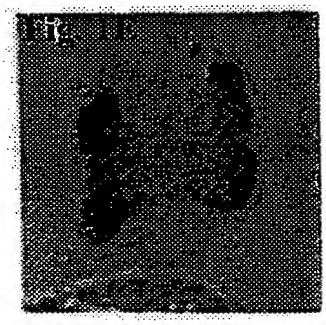


Fig. 1F

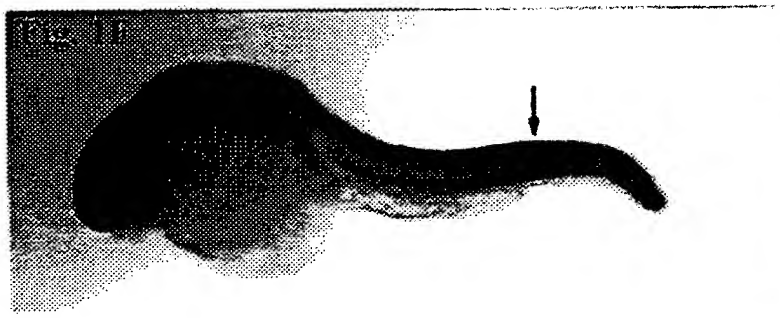


Fig. 1G

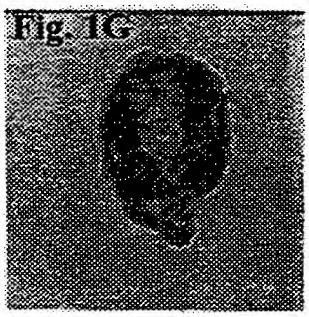


Fig. 1H

Fig. 1I

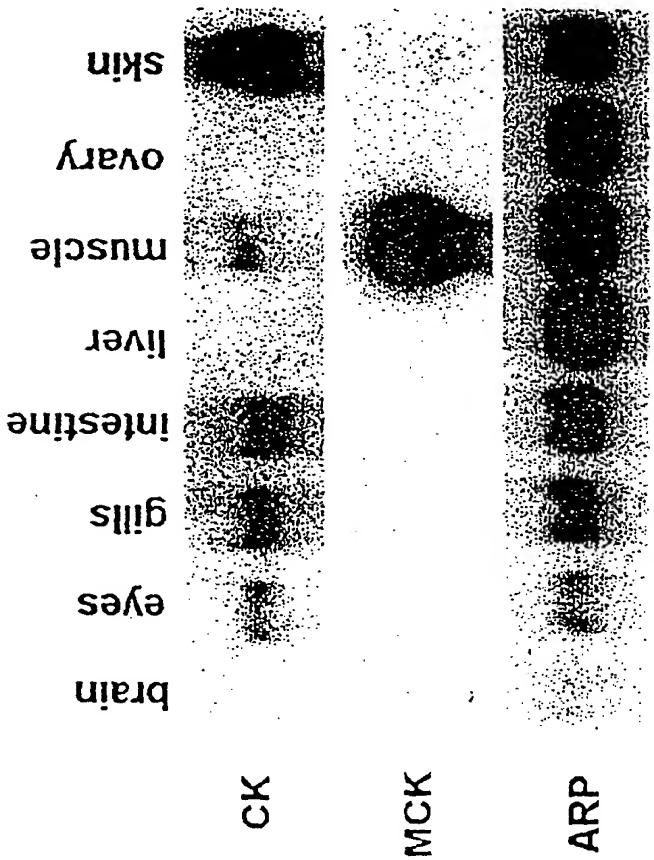
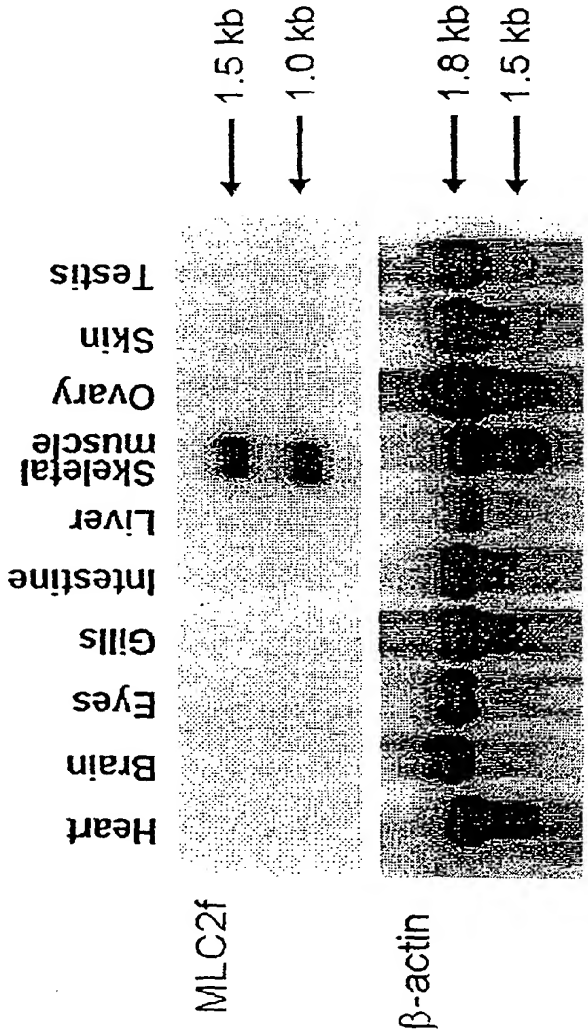

CK
MCK
ARP
brain
eyes
gills
intestine
liver
muscle
ovary
skin

Fig. 2A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1.0 kb
1.8 kb
1.5 kb
Heart
Brain
Eyes
Gills
Intestine
Liver
Skeletal muscle
Ovary
Skin
Testis
MLC2f
β-actin

Fig. 2B

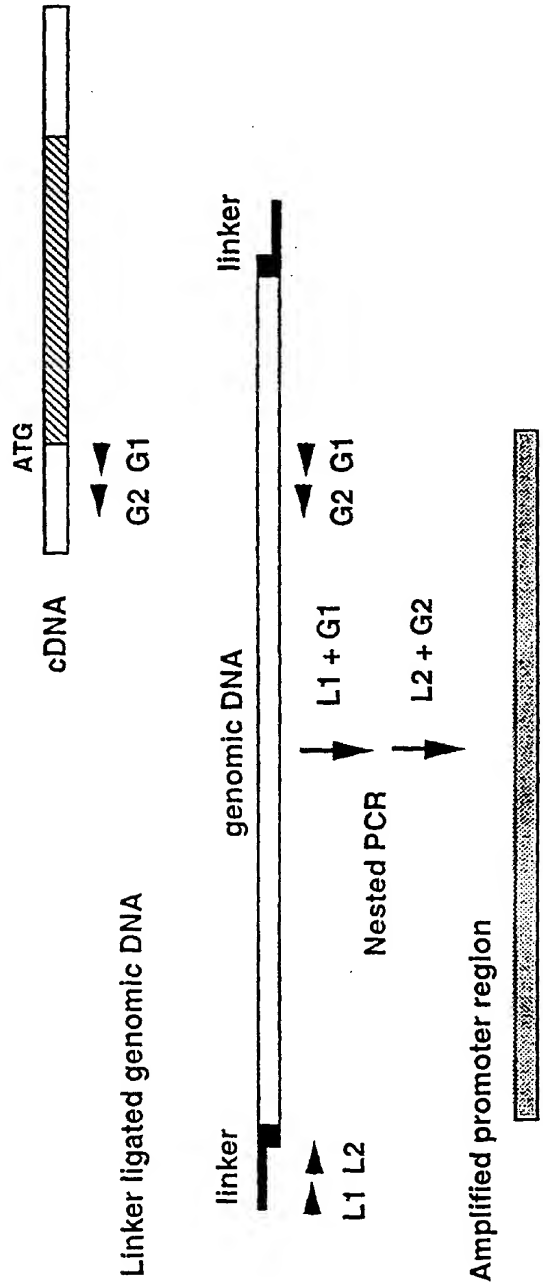

ATG
G2 G1
cDNA
linker
genomic DNA
G2 G1
L1 + G1
L2 + G2
Nested PCR
linker
L1 L2
Linker ligated genomic DNA
Amplified promoter region

Fig. 3

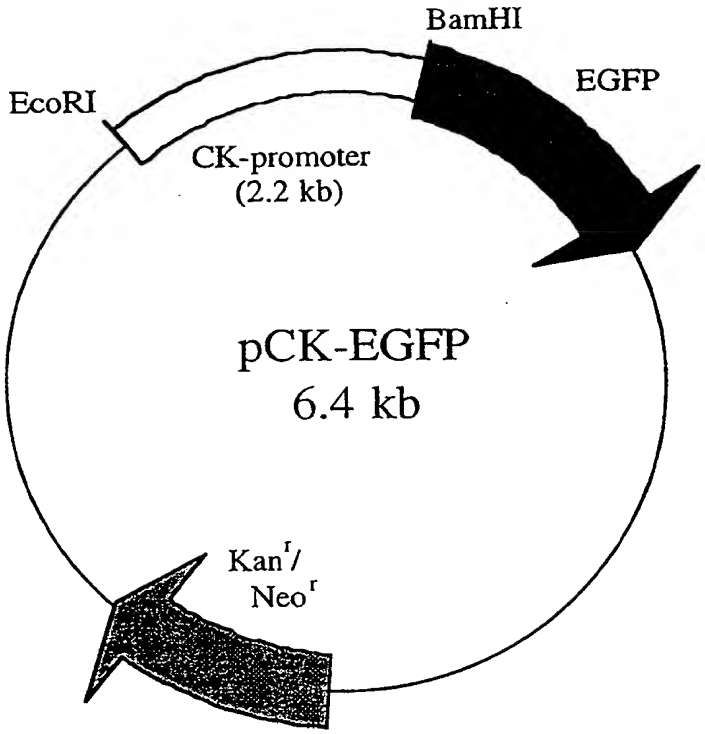
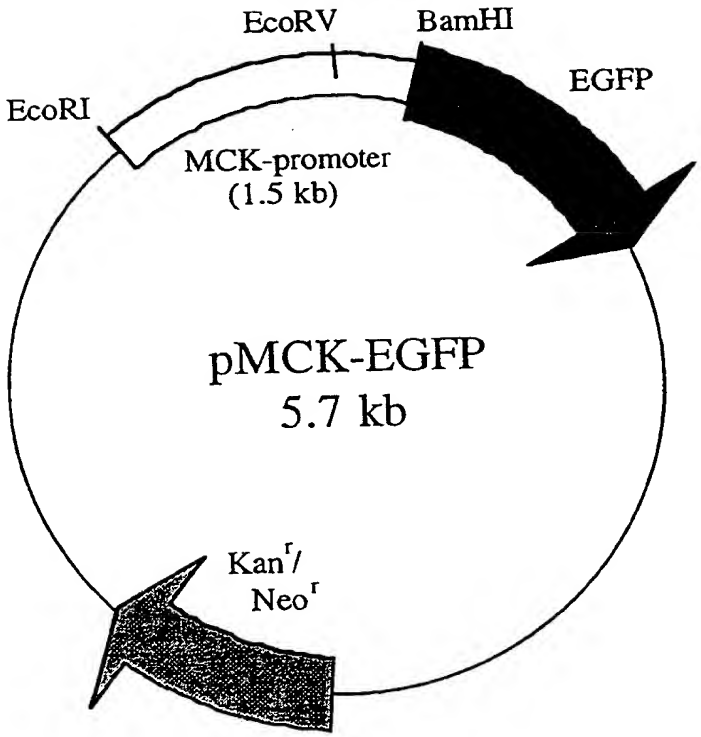


Fig. 4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EcoRI
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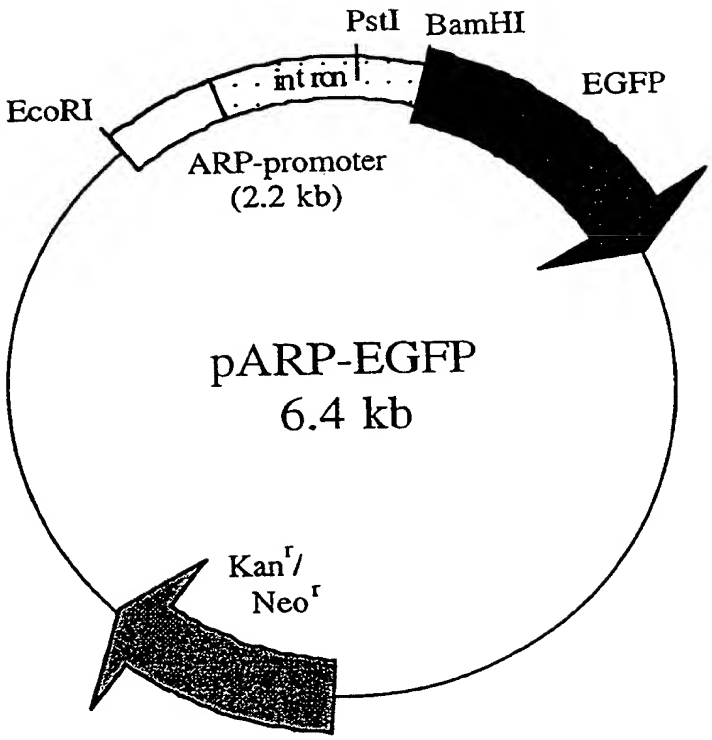
pMCK-EGFP
5.7 kb
Kan^r/
Neo^r

Fig. 5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EGFP
intron
EcoRI
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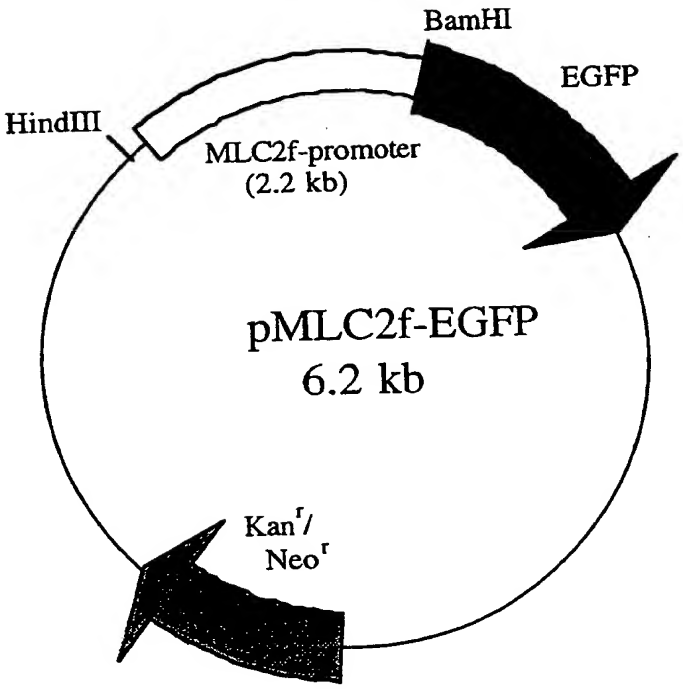
pARP-EGFP
6.4 kb
Kan^r/
Neo^r

Fig. 6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HindIII
MLC2f-promoter
(2.2 kb)
pMLC2f-EGFP
6.2 kb
Kan^r/
Neo^r

Fig. 7

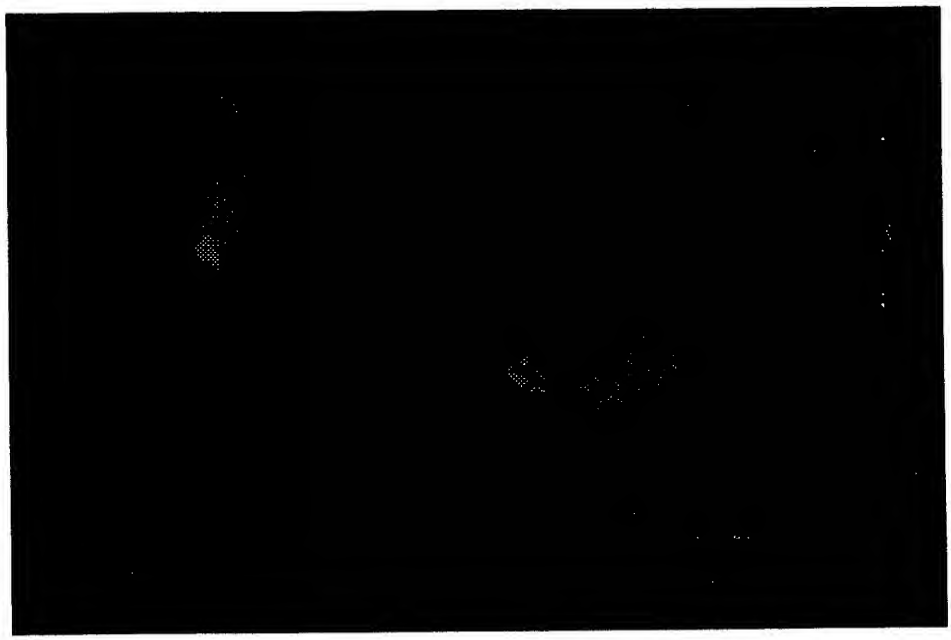


Fig. 8

Fig. 9

Fig. 10

Fig. 11A

Fig. 11B

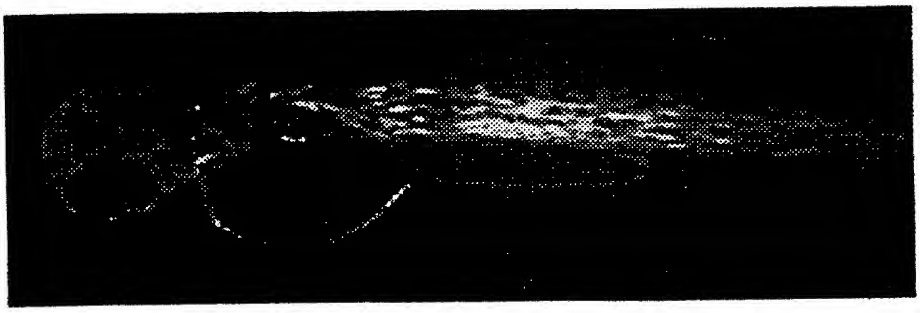
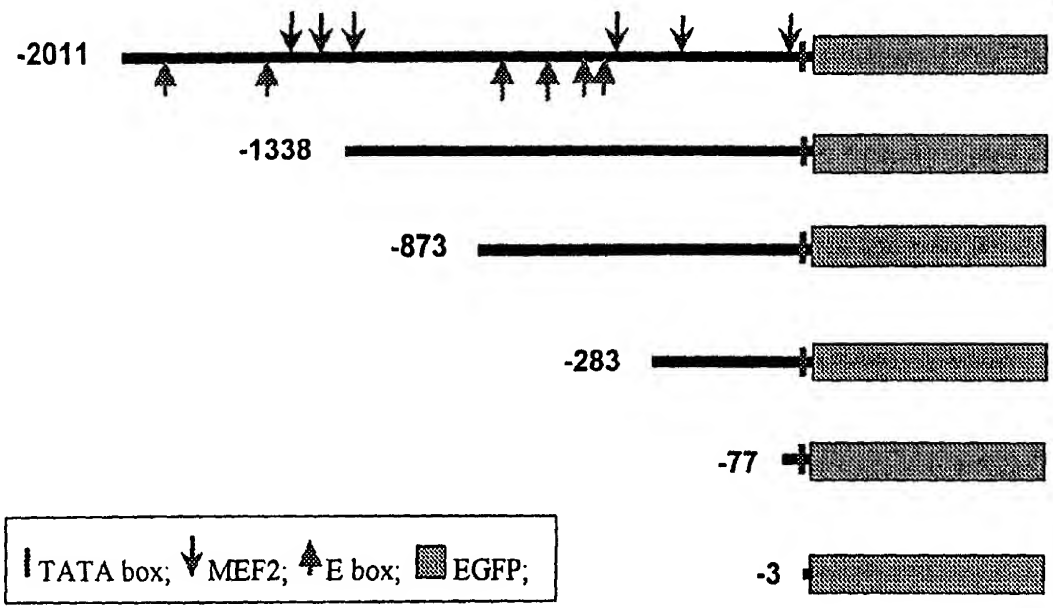


Fig. 12A

Fig. 12B

Fig. 12C

Fig. 13



	Exp. in skeletal muscles (%)	Levels of Exp. in Skeletal muscles (%)		
		S	M	W
-2011	100	23	37	40
-1338	100	14	23	63
-873	100	2	30	68
-283	100	0	15	85
-77	95.7	0	11	89
-3	46.6	0	0	100

S, Strong Expression; M, Medial Expression;
W, Weak Expression

INTERNATIO SEARCH REPORT

Inte[redacted]al application No.
PCT/SG 99/00079

A. CLASSIFICATION OF SUBJECT MATTER

IPC[7]: C 12 N 15/12, 5/16; C 12 Q 1/66, 1/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC[7]: C 12 N 15/12, 5/16; C 12 Q 1/66, 1/48

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, PAJ, CAS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 98/56902 A2 (MEDICAL COLLEGE OF GEORGIA RESEARCH INSTITUTE, INC.) 17 December 1998 (12.12.98) abstract; claims 1-9, 19, 20, 25-30.	1-8, 13-18
A	WO 96/03034 A1 (MASSACHUSETTS INSTITUTE OF TECHNOLOGY) 08 February 1996 (08.02.96) page 2; claims 1, 2, 9, 10, 29-35.	1-8, 14-16
A	WO 9815627 A1 (UNIVERSITY OF SOUTHAMPTON) 16 April 1998 (16.04.98) claims 1, 2, 5, 6, 11-14, 20-29.	1-8
A	MULLER et al. "Activator effect of coinjected enhancers on the muscle-specific expression of promoters of zebrafish embryos" Mol. Reprod. Dec. 1997, 47(4), 404-412 (Eng). Chem. abstr. Vol. 127, No. 10, 08 September 1997 (08.09.97) (Columbus, Ohio, USA) page 195, right column, the abstract No. 131871y	2,3,5-7

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
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„O" document referring to an oral disclosure, use, exhibition or other means
„P" document published prior to the international filing date but later than the priority date claimed
„T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
„X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
„Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
„&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 November 1999 (12.11.99)	13 December 1999 (13.12.99)
Name and mailing adress of the ISA/AT Austrian Patent Office Kohlmarkt 8-10; A-1014 Vienna Facsimile No. 1/53424/200	Authorized officer Mosser Telephone No. 1/53424/437

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/SG 99/00079

Im Recherchenbericht angeführtes Patentdokument Patent document cited in search report Document de brevet cité dans le rapport de recherche	Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets	Datum der Veröffentlichung Publication date Date de publication
WO A2 9856902	17-12-1998	AU A1 79558/98 WO A3 9856902	30-12-1998 04-03-1999
WO A1 9603034	08-02-1996	keine - none - rien	
WO A1 9815627	16-04-1998	EP A1 931143 GB A0 9621113	28-07-1999 27-11-1996